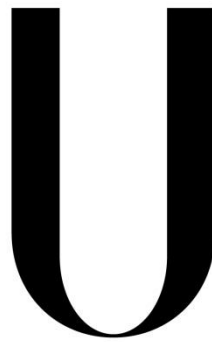


Universidade de Lisboa

Faculdade de Farmácia

Departamento de Microbiologia e Imunologia



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Interference of *Mycobacterium tuberculosis* with the endocytic/ antigen
presentation pathways on macrophages and dendritic cells

David Alexandre Rodrigues Pires

Doutoramento em Farmácia

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Tese orientada pela Professora Doutora Elsa Maria Ribeiro dos Santos Anes e co-orientada pelo Professor Doutor Olivier Neyrolles, especialmente elaborada para a obtenção do grau de doutor em Farmácia na especialidade de Biologia Celular e Molecular,

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Preface

This thesis encompasses the work I produced from 2010 to 2014 while integrated in Prof. Elsa Anes' group and in collaboration with my colleagues at Unidade de Retrovírus e Infecções Associadas of Faculdade de Farmácia da Universidade de Lisboa. A portion of the results were produced in collaboration with three other research groups: Prof. Olivier Neyrolles group from the Institute of Pharmacology and Structural Biology of Université Paul Sabatier at Toulouse, France; Prof. Miguel Viveiros' group from Instituto de Higiene e Medicina Tropical of Universidade Nova de Lisboa, Portugal; Prof. Michael Niederweis' group from the Department of Microbiology of The University of Alabama at Birmingham, USA. This thesis is the culmination of several different projects and collaborations that were brought together by their common goal to unravel the underlying mechanisms involved in the ability of the etiologic agent of tuberculosis, *Mycobacterium tuberculosis*, to persist in the human host by sustaining or manipulating the intracellular bactericidal response of macrophages and dendritic cells.

I begin this thesis by introducing the disease and the complex steps that occur throughout the endocytic pathways, spanning from: recognition and uptake of the pathogen; response produced; regulatory events and the physiology of the intervening cells. All these processes are deeply interconnected, and while complex I felt the need to provide the readers with a broad review, touching all of them, even if, in some cases, only lightly.

The 4 chapters that follow the introduction represent original scientific studies that are already under review for publishing or will be submitted. Chapters 2 and 3 focus on the function and regulation of cathepsins. Chapter 3 centers on the use of pharmacologic compounds to manipulate the endocytic pathways in favor of the host. Chapter 4 focuses on the membrane of mycobacteria as a barrier against the aggressions of the host or pharmacological therapy.

I end the thesis with a brief conclusion that covers and interconnects the several chapters and employed strategies.

During the time span covered by this thesis I presented and authored the following communications and publications related to this thesis or to other fields of study of tuberculosis:

Research Articles*

Danilchanka O, Pires D, Anes E, and Niederweis M. The *Mycobacterium tuberculosis* outer membrane channel protein CpnT confers susceptibility to toxic molecules. Antimicrobial Agents and Chemotherapy, 2014 (under review).

Bettencourt P, Marion S, **Pires D**, Santos LF, Lastrucci C, Carmo N, Blake J, Benes V, Griffiths G, Neyrolles O, Lugo-Villarino G, Anes E. Actin-binding protein regulation by microRNAs as a novel microbial strategy to modulate phagocytosis by host cells: the case of N-Wasp and miR-142-3p. Frontiers in Cellular and Infection Microbiology, 2013.

Luo X, Pires D, Aínsa JA, Gracia B, Duarte N, Mulhovo S, Anes E[&], Ferreira MJ[&]. Zanthoxylum capense constituents with antimycobacterial activity against *Mycobacterium tuberculosis* in vitro and ex vivo within human macrophages. Journal of Ethnopharmacology, 2013.

Luo X, Pires D, Aínsa JA, Gracia B, Mulhovo S, Duarte A, Anes E, Ferreira MJ. Antimycobacterial evaluation and preliminary phytochemical investigation of selected medicinal plants traditionally used in Mozambique. Journal of Ethnopharmacology, 2011

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Book Chapters*

P. Bettencourt, D. Pires, N. Carmo and E. Anes. Application of Confocal Microscopy for Quantification of Intracellular Mycobacteria in Macrophages. Microscopy: Science, Technology, Applications and Education, 2010.

Publications in Conference Proceedings*

Ferreira MJ, Luo X, Pires D, Ainsa JA, Gracia B, Duarte N, Mulhovo S, Anes E. Searching for natural antituberculars from mozambican medicinal plants. Instituto de Investigação Científica Tropical, 2013.

Pires D, Bettencourt P, Carmo, N, Bergant, T, Jordao L and Anes, E. Interference of Mycobacterium tuberculosis with the endocytic pathways on macrophages and dendritic cells from healthy donors: role of Cathepsins. Drug Discovery Today, 2010.

Pires P, Bettencourt P, Carmo N, Niederweis M and Anes, E. Role of Mycobacterium tuberculosis outer-membrane porins in bacterial survival within macrophages . Drug Discovery Today, 2010.

Communications in Scientific Meetings*

Pires D, Pombo J, Marques J, Carmo N, Bettencourt P, Anes E. Oral communication “*Mycobacterium tuberculosis* regulates cathepsin S activity by manipulating the expression of miR-106b” presented at the “6th iMed.UL Postgraduate Students Meeting”, 2014, Lisbon, Portugal.

* first authors

Pires D, Pombo J, Marques J, Carmo N, Bettencourt P, Anes E. Oral communication “*Mycobacterium tuberculosis* manipulates host cathepsins to increase its survival and lower the expression of antigen presentation molecules” presented at the “Lisbon Workshop on New Strategies to Fight Tuberculosis”, 2014, Lisbon, Portugal.

Machado D, **Pires D**, Perdigão J, Ramos J, Couto I, Portugal I, Anes E and Viveiros M. Oral and poster communication “Efflux inhibitors against drug resistant *Mycobacterium tuberculosis*: antimicrobial agents and enhancers of macrophage killing activity” presented at the “35th Annual Congress of the European Society of Mycobacteriology”, 2014, Vienna, Austria.

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Pires D, Carmo N, Marques J, Pombo J, Timóteo P, Bettencourt P, and Anes E. Poster communication “Cut or be Cut: Cathepsins and their Inhibitors during mycobacterial infection of Macrophages and Dendritic Cells” presented at the “5th iMed.UL Postgraduate Students Meeting”, 2013, Lisbon, Portugal.

Pires D. Oral communication “To control or to be controlled during *M. tuberculosis* infection? Cathepsins and their inhibitors in TB immune response” presented at “VI Ciclo de Scienceshops: Tuberculose”, 2013, Lisbon, Portugal.

Ferreira MJ, Luo X, **Pires D**, Ainsa JA, Gracia B, Duarte N, Mulhovo S, Anes E, Mulhovo S, Anes E. Conference proceedings “Searching for Natural

Antituberculars from Mozambican Medicinal Plants” presented at “Congresso Internacional Saber Tropical em Moçambique: História, Memória e Ciência”, 2012, Lisbon, Portugal.

Pires D, Carmo N, Marques J, Bugalhão J, Bettencourt P, Anes E. Poster communication “To control or to be controlled during *M. tuberculosis* infection? Cathepsins and their Inhibitors within Host Dendritic Cells or Macrophages” presented at the EMBO Conference Tuberculosis 2012: Biology, pathogenesis, intervention strategies, 2012, Paris, France.

Carmo N, **Pires D**, Bugalhão J, Marques J, Bettencourt P, Timóteo P, Anes E. Poster communication “Host factors affecting *Mycobacterium tuberculosis* survival within macrophage” presented at the EMBO Conference Tuberculosis 2012: Biology, pathogenesis, intervention strategies, 2012, Paris, France.

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Luo X, **Pires D**, Aínsa JA, Gracia B, Mulhovo S, Duarte A, Anes E, Ferreira MJ. Poster communication “Phytochemical characterization of antimycobacterial crude extracts from medicinal plants traditionally used in Mozambique”

presented at the “59th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research”, 2011, Antalya, Turkey.

Pires D, Bettencourt P, Carmo N, Bergant T, Jordão L, Anes E. Poster communication "Cathepsins B and S Content in Mycobacterium Containing Compartments in Human Macrophages and Dendritic cells" presented at the “Current Opinion in Cellular Host-Pathogen Interactions”, 2010, Amsterdam, The Netherlands.

Pires D, Bettencourt P, Carmo N, Bergant T, Jordao L, Anes E. Poster communication "Interference of Mycobacterium tuberculosis with the endocytic pathways on macrophages and dendritic cells from healthy donors: role of Cathepsins" presented at the 3rd International Symposium "Celular Delivery of Therapeutic Macromolecules 2010", Cardiff, United Kingdom.

Pires D, Bettencourt P, Carmo N, Niederweis M, Anes E. Poster communication "Role of Mycobacterium tuberculosis outer-membrane porins in bacterial survival within macrophages" presented at the 3rd International Symposium "Celular Delivery of Therapeutic Macromolecules 2010", Cardiff, United Kingdom.

Bettencourt P, Marion S, **Pires D**, Blake J, Benes V, Griffiths G, Anes E. Poster communication "MicroRNAs are Involved in Early Steps of Mycobacteria Infection on Macrophages" presented at the “2nd Host Genetics Control of Infectious Diseases”, 2009, Paris, France.

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Every time I end a cycle in my life I wonder if I have learnt anything useful from it. Most times, it takes facing a new challenge to truthfully realize the value of the experiences we have lived. Right now, I have absolutely no doubts regarding the value of this period of my life and thus, there is no lack of people to thank.

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Specific acknowledgments to FCT funding and other institutions are mentioned at the end of each chapter, when required.

Abstract

Tuberculosis is a worldwide health concern, caused by the bacterium *Mycobacterium tuberculosis* (MTB) that causes the death of approximately 1.3 million people per year making it the deadliest bacterial infection for human beings. Although antibiotic treatment exists, its severity and long duration hampers the implementation and compliance, leading to the generation of multi-resistant strains. This thesis proposes that by enhancing the cellular immunity against the bacteria we can improve the therapeutic and reduce the emergence of resistant strains. In order to test this hypothesis, we first explored the regulation of lysosomal cathepsins caused by MTB to identify key molecules being subverted by the pathogen. Cathepsins are major proteolytic enzymes active in the endocytic pathways and thus, are interesting targets to study in the context of an intracellular pathogen such as MTB. Then, it is revealed that MTB regulates one of these cathepsins, cathepsin S, by manipulating the expression of the micro-RNA, miR-106b-5p. This identified one potential target to counter MTB-mediated manipulation of the host cells. Furthermore, it is shown that pharmacologic drugs already approved for other pathologies can be used to alter the phagosomal environment and improve macrophage-mediated killing of MTB. Finally it is shown that restriction of outer-membrane transport is a resistance factor of MTB and a novel outer-membrane channel is an entry pathway for drugs and host microbicidal molecules that may be exploited in the future. Altogether, we show that we can improve host cell-mediated antimycobacterial mechanisms and we propose several molecules that may be targeted for this purpose.

Keywords

Tuberculosis, *Mycobacterium tuberculosis*, macrophage, dendritic cell, cathepsins, micro-RNAs, ion channel blockers, outer-membrane channel, drug-resistance.

Resumo*

A tuberculose é um flagelo à escala mundial causando a morte de mais de 1 milhão de pessoas anualmente e possuindo uma prevalência registada em cerca de 12 milhões de pessoas em 2012. A doença é causada por bactérias do complexo *Mycobacterium tuberculosis* que compreende um conjunto de espécies do género *Mycobacterium* com elevada proximidade filogenética e que têm em comum serem agentes causadores de tuberculose, apesar apresentarem tropismo para hospedeiros distintos. A espécie *Mycobacterium tuberculosis* (MTB) é a principal responsável para tuberculose humana. Estas bactérias são aeróbicas, com forma bacilar e com um longo tempo de geração entre 12 a 24 h. Uma das suas características estruturais mais distintas é a sua parede e membrana externa. Estas constituem uma forte barreira contra compostos tóxicos, quer de origem farmacológica ou provenientes do sistema imunitário do hospedeiro. É compreendida por uma parede de peptidoglicano e arabinogalactano localizada no espaço periplasmático, ligada aos ácidos micólicos do folheto interno da membrana externa que também compreende no seu folheto externo um conjunto de glicolípidos. É também na membrana de MTB que se localizam vários factores de virulência e resistência, tal como os sistemas de secreção tipo VII, que provocam a necrose das células dos hospedeiro, bombas de efluxo que permitem a extravasão de compostos microbicidas ou de fármacos e canais selectivos ou em número reduzido que restringem a entrada destes mesmos compostos e fármacos.

A patogénese da tuberculose normalmente inicia-se com a inalação dos bacilos para os pulmões e a consequente fagocitose por células residentes nos alvéolos como os macrófagos alveolares. A infecção leva à libertação de citocinas pró-inflamatórias, quimiocinas e factores de adesão celulares que resultam na migração de outras células imunitárias para o local de infecção, tal como células dendríticas em circulação no sangue. Apesar da incerteza quanto à classificação dos vários tipos de células intervenientes na imunidade celular contra bactérias, vários estudos sugerem um papel local para os macrófagos,

* extended thesis abstract in Portuguese

fagocitando os invasores e participando na sua destruição através dos mecanismos degradativos das vias endocíticas como a libertação de espécies reactivas oxidativas, acidificação do fagossoma e fusão com lisossomas para a entrega de proteases e lipases que irão degradar e processar antígenos da bactéria. No tecido infectado os macrófagos irão apresentar estes antígenos a linfócitos T, estimulando a resposta imune adaptativa. No entanto, são as células dendríticas que se pensam serem as principais iniciadoras da imunidade adaptativa. Ao fagocitarem a bactéria, migram para os nódulos linfáticos sofrendo um processo de maturação ao longo da migração. Este processo resultará no aumento da expressão de moléculas apresentadoras de antígenos HLA classe II que irão estimular células T específicas para os antígenos da bactéria. No tecido infectado, o conjunto de macrófagos, células dendríticas e linfócitos dará origem à estrutura mais evidente da tuberculose, o granuloma. Aqui, a infecção está contida, existindo um equilíbrio entre o crescimento da população de bacilos e a sua destruição pelas células do hospedeiro. Desequilíbrios causados por supressão da imunidade são a principal causa para a rotura do granuloma e consequente libertação de bacilos nos pulmões que serão expelidos pelas vias respiratórias infectando novos indivíduos.

Uma das características mais estudadas do bacilo da tuberculose é a sua capacidade para persistir nas células do hospedeiro, resistindo e subvertendo os mecanismos degradativos intracelulares dos fagócitos, mencionados anteriormente. MTB inibe a maturação do fagossoma e fusão com os lisossomas evitando a acidificação e activação de enzimas proteolíticas como as catepsinas. As catepsinas são hidrolases lisossomais que na maioria dos casos necessitam de um ambiente ácido para manterem uma estrutura estável e serem activadas. A regulação das catepsinas também é feita por inibidores endógenos, as cistatinas, que bloqueiam de forma reversível a sua zona catalítica. As catepsinas intervêm em vários processos de homeostasia celular, participando na degradação de componentes membranares e organelos a serem reciclados; no processamento de receptores e outras enzimas de forma a adquirirem uma estrutura funcional; na desencadear de processos de morte celular; no processamento de péptidos para apresentação

a células T ou no processamento da própria maquinaria apresentadora de antígenos.

As várias características que permitem a MTB sobreviver e ocupar nichos nas células do hospedeiro e no granuloma e resistir a fármacos antibióticos resultam no facto de o tratamento da tuberculose ser moroso e agressivo para o hospedeiro, com uma terapêutica que dura em média 6 meses. Isto leva a uma grande dificuldade por parte dos sistemas de saúde em acompanhar o tratamento dos doentes e garantir uma correcta posologia. Estas dificuldades resultaram na emergência de várias estirpes resistentes e multi-resistentes que dificultam o tratamento, aumentam a mortalidade e podem eventualmente tornar-se extremamente resistentes ou totalmente resistentes, deixando portanto de existir terapêutica adequada para estes últimos. Perante este problema surge a necessidade de novas propostas e estratégias que evitem o surgimento de resistências ao abordar o problema por múltiplas vias. Nesta tese, proponho que através da identificação dos factores do hospedeiro que estão a ser manipulados pela bactéria e da identificação dos seus próprios factores de resistência podemos desenhar estratégias que visem aumentar a capacidade das células do hospedeiro em eliminar os bacilos. Esta estratégia pode ser empregada sinergisticamente com a terapêutica antibiótica aumentando significativamente a probabilidade de sucesso e limitando a emergência de resistências.

O problema foi abordado numa primeira fase através da descrição da modulação das catepsinas do hospedeiro por MTB em macrófagos e células dendríticas. Demonstra-se que no geral, a infecção por MTB resulta na sub-regulação ou inibição da sobre-regulação que é desencadeada durante infecção por micobactérias não patogénicas. É também evidenciado que esta inibição da expressão das catepsinas é favorável ao crescimento intracelular da bactéria e que curiosamente resulta também num aumento da expressão de moléculas apresentadoras de antígenos HLA classe II.

A abordagem seguinte passou por identificar mecanismos de regulação das catepsinas ou do ambiente fagossomal em geral, de forma a encontrar alvos ou estratégias que permitam reverter a modulação causada por MTB. O primeiro

mecanismo encontrado foi a regulação da catepsina S por um micro-RNA do hospedeiro, miR-106b-5p. Os micro-RNAs são pequenos RNAs que com conjunto com um complexo de silenciamento inibem a tradução de mRNAs específicos, silenciando a expressão desses genes. Neste caso, a catepsina S foi uma das catepsinas identificadas como importantes no controlo da infecção e na apresentação de antígenos. Ao identificar-se este micro-RNA como uma molécula manipulada por MTB para controlar a expressão da catepsinas S encontramos uma via potencial para reverter esta regulação e fortalecer a resposta do hospedeiro. O segundo mecanismo encontrado foi a manipulação do ambiente fagossomal através do uso de fármacos aprovados para patologias distintas da tuberculose que possuem actividade bloqueadora de bombas de efluxo. Foi evidenciado que estas moléculas actuavam na bactéria inibindo os seus sistemas de efluxo e portanto restringindo esta via de resistência a antibióticos, mas também alterando inespecificamente as vesículas endocíticas do hospedeiro resultando na sua acidificação. Demonstra-se também que essa acidificação resulta no aumento da actividade da catepsina B, sugerindo que neste ambiente as catepsinas que possuem maior actividade em ambientes acídicos estão mais activas. No seu todo, esta estratégia permite melhorar o tratamento antibiótico contra estirpes de MTB susceptíveis e resistentes, sendo um forte indicador de uma potencial via alternativa de terapêutica contra a emergência de estirpes multi-resistente de MTB.

Finalmente, é colocado o foco na bactéria e nos seus factores de resistência, especificamente no transporte de pequenas moléculas por canais na membrana externa. É revelado que a um canal formado pela proteína CpnT, recentemente descrito, é uma via de susceptibilidade ao constituir uma porta de entrada para fármacos e moléculas bactericidas do hospedeiro, tal como o óxido nítrico. MTB restringe esta via de entrada exprimindo poucos canais na membrana externa, no entanto estes são necessários para a o influxo de nutrientes e por isso podem ser potencialmente explorados no desenvolvimento no novas terapêuticas.

Concluindo, nesta tese: (1) identifico moléculas do hospedeiro manipuladas por MTB, as catepsinas; (2) Identifico vias de regulação destas moléculas que

podem ser usadas para fortalecer o hospedeiro, os micro-RNAs e bloqueadores de bombas de efluxo; (3) identifique uma via de susceptibilidade na bactéria, a proteína canal CpnT.

Palavras-chave

Tuberculose, *Mycobacterium tuberculosis*, macrófagos, células dendríticas, catepsinas, micro-RNAs, bloqueadores de canais iônicos, canais da membrana externa, resistência a antibióticos.

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List of abbreviations

AP-1	activator protein-1
APC	antigen presenting cell
ATP	adenosine triphosphate
BCG	bacillus Calmette–Guérin
Cat	cathepsin
CD	complex of differentiation
CIITA	class II transactivator
CLIP	class II-associated li peptide
CPZ	chlorpromazine
CR	complement receptor
DC	dendritic cell
DC-SIGN	DC-specific intracellular adhesion molecule 3–grabbing nonintegrin
ECM	extracellular matrix
EEA1	early endosomal autoantigen 1
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
EU/EEA	European Union/ European Economic Area
FPX	flupenthixol
GAP	GTPase-activating protein
GMDP	N-glycolylated form of muramyl dipeptide
GTPase	guanosine triphosphatase
HAL	haloperidol
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
IFN-γ	interferon-gamma
IGRA	IFN-γ reactivity essay
IL	interleukin
imDC	immature DC
iNOS	inducible nitric oxide synthase
IRAK	interleukin-1 receptor-associated kinase
LM	lipomannan
LPS	lipopolysaccharide
LRR	leucine rich region
LXA4	lipoxin A4
ManLAM	mannosylated lipoarabinomannan
MAPK	mitogen-associated protein kinase
MDR-TB	multi-drug resistant tuberculosis

MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
miRNA	micro RNA
MMR	macrophage mannose receptor
mRNA	messenger RNA
Msm	<i>Mycobacterium smegmatis</i>
MTB	<i>Mycobacterium tuberculosis</i>
MTC	<i>Mycobacterium tuberculosis</i> complex
mTOR	mammalian target of rapamycin
NADPH	nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor-kappa B
NK	natural killer
NLR	nod-like receptor
NOD	nucleotide oligomerization domain
NOS2	nitric oxide synthase 2
NTD	N-terminal domain
PAMP	pathogen associated molecular pattern
PAS	<i>p</i> -aminosalicylic acid
PGE2	prostaglandin E2
PI3K	phosphatidylinositol 3-kinase
PI3P	phosphatidylinositol-3 phosphate
PIM	phosphatidylinositol mannan
PPAR γ	peroxisome proliferator-activated receptor γ
PPD	purified protein derivate
PRR	pattern recognition receptor
RANTES	regulated on activation, normal T cell expressed and secreted
RD	region of differentiation
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNS	reactive nitrogen species
ROS	reactive oxygen species
rRNA	ribosomal RNA
siRNA	small interfering RNA
SNARE	soluble N-ethylmaleimide-sensitive fusion protein attachment receptor
SNP	sodium nitroferricyanide (III) dehydrate
SP	surfactant protein
sRNA	small RNA
TB	tuberculosis

TDM	trehalose dimycolate
TGF- β	transforming growth factor-beta
Th1	T-helper 1
TipDC	TNF- α /iNOS-producing DC
TLR	toll-like receptor
TNF- α	tumor necrosis factor-alpha
TZ	thioridazine
vATPase	vacuolar adenosine triphosphatase
VP	verapamil
WHO	World Health Organization
XDR-TB	extremely-drug resistant tuberculosis

Chapter 1: Introduction

On the 24th of March of 1882, Robert Koch presented his work to the Physiological Society of Berlin (Koch, 1882), a presentation that made a deep impact in the scientific field of infection - which was just starting to emerge. During that exposition, Koch presented the evidence for the isolation of a living rod-shaped organism causing the disease, tuberculosis:

“On the basis of my extensive observations, I consider it as proven that in all tuberculous conditions of man and animals there exists a characteristic bacterium which I have designated as the tubercle bacillus, which has specific properties which allow it to be distinguished from all other microorganisms.”

Moreover, he was able to show that not only could he identify the bacillus and stain infected tissues; he could also recover and cultivate it in the laboratory and use it to cause the disease in healthy animals:

“In order to prove that tuberculosis is brought about through the penetration of the bacilli, and is a definite parasitic disease brought about by the growth and reproduction of these same bacilli, the bacilli must be isolated from the body, and cultured so long in pure culture, that they are freed from any diseased production of the animal organism which may still be adhering to the bacilli. After this, the isolated bacilli must bring about the transfer of the disease to other animals, and cause the same disease picture which can be brought about through the inoculation of healthy animals with naturally developing tubercle materials.”

The identification of a microorganism causing a disease opened an array of possibilities for medical research. Causative agents could now be isolated and tested in order to develop treatment. More than that, a specific set of criteria had been drawn by Koch's postulates (Koch, 1884) creating a well-defined method for the validation of infectious microorganism. These achievements were, by themselves, worthy of scientific praise, yet the fact that they were performed on the world's greatest killer at that time (Barnes, 2000), were all the more significant. In 1905, Robert Koch was awarded the Nobel Prize for Physiology or Medicine, adding to several other awards and distinctions received during his lifetime.

1.1. Tuberculosis

Tuberculosis is the disease caused by bacteria of the *Mycobacterium tuberculosis* Complex (MTC), a group comprising the closely related species *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae* and *M. canettii* (Coscolla et al., 2013; Wirth et al., 2008). This disease affects humans and other mammals although host tropism varies within the MTC. The majority of infected individuals develop the disease in the lungs (pulmonary TB) although the disease may occur in any other organ (extrapulmonary TB) (ECDC and WHO, 2014). The spread of the bacilli is most effective when the disease affects the lungs, since it uses the respiratory system to escape the host through coughing and then to infect other individuals (Sepkowitz, 1996). The main symptoms of the disease are a long-lasting cough (3 weeks or longer), pain in the chest, hemoptysis or expectoration, fatigue, weight loss, anorexia, chills, fever and nocturnal hyperhidrosis (CDC, 2013).

The main method of diagnosing the disease has remained almost unmodified in the last 100 years, relying on microscopic observation of sputum smear samples to identify the presence of the bacilli and then cultivation of the sample which takes several weeks to yield results and depends on developed laboratory infrastructure and trained personal. New molecular methods, such as Xpert MTB/RIF, have recently been applied to the diagnosis allowing much faster detection rates. The World Health Organization's (WHO) current effort to increase the widespread use of Xpert MTB/RIF has enabled rapid diagnosis of pulmonary tuberculosis and detection of resistance to rifampicin, the most powerful drug used for treatment, by targeting the *rpoB* gene (Raizada et al., 2014; Zar et al., 2013).

Tuberculosis is a fatal disease when untreated, with estimates of 70% mortality for smear-positive and 20% for culture-positive smear-negative untreated tuberculosis patients (Tiemersma et al., 2011). The causes of death often involve severe necrosis of the infected tissues resulting in chronic respiratory failure and hemoptysis (Davis et al., 1985; Tomono, 1998). However, the majority of current deaths related to TB could be prevented using the standard antibiotic therapy. The first line therapy involves the use of an antibiotic cocktail

consisting of rifampicin, isoniazid, ethambutol and pyrazinamide during 6 months. This regimen is reported to yield 85% chance of a successful treatment (WHO, 2013a). Tuberculosis caused by multi-drug resistant strains (MDR-TB), however, has a severe impact in treatment success rates, lowering them to below 50%, on the global scale. MDR-TB is caused by bacilli resistant to the two most effective drugs, rifampicin and isoniazid, and occurs in 3.6% of new TB cases and 20% in previously TB-treated cases (WHO, 2013a). Treatment of MDR-TB involves a longer therapy of 20 months, as recommended by the WHO, using more toxic and expensive second line drugs. The high incidence of MDR-TB in recurring TB cases is an indication that a significant part of the occurrences could be prevented by focusing on treatment monitoring and compliance. Amongst TB cases caused by bacilli resistant to treatment, extreme cases are classified as extremely drug-resistant TB (XDR-TB), when the bacteria are resistant to rifampicin, isoniazid, any fluoroquinolone and to at least one of the second line injectable drugs: kanamycin, capreomycin or amikacin. In these extreme cases, successful treatment rates may drop to 20%.

Concerning vaccination, although new candidates and strategies are being put to trial, none so far was proved to induce protective immunity in the host (Kaufmann, 2014). The only vaccine still administered in numerous regions of the world is the famous Bacille Calmette-Guérin (BCG) vaccine. BCG is an attenuated strain from over a hundred years ago, derived from continuous culture of *M. bovis* for over a decade. This led to the loss of several virulence associated regions of the genome with the resulting consequence of also losing important antigenic regions (Zhang et al., 2013b), especially the RD1 region which encodes several virulence factors. The vaccine is able to induce a strong immunological response in the host but ineffectively protects against adult pulmonary TB (Fine, 1995) and is mostly used to prevent severe forms of TB in children. However, new guidelines recommend avoiding treatment of HIV-infected children following studies indicating that immune-compromised children may suffer disseminated BCG disease (Hesseling et al., 2007).

Mycobacterium tuberculosis is estimated to infect one third of the human population although the estimates vary depending on the region, for example, in the United States of America, only 5% of individuals are expected to be infected

(CDC, 2013). Still, only a small fraction, ranging from 5% to 10% of infected individuals, will develop disease, which in 2012 resulted in 8.6 million new cases. Tuberculosis prevalence is unevenly distributed, with regions such as southwest Asia and western pacific accounting for more than half of total cases and Africa accounting for one-quarter of cases (WHO, 2013a). Worldwide, approximately 12 million individuals suffer from TB. The probability of developing the disease is much higher in HIV-infected individuals with a 12% prevalence of HIV in TB incident cases worldwide, rising to 36.6% in Africa and decreasing in Europe to 5.26% in 2012 (WHO, 2013a). TB is also more prevalent in males than females, with more incidence in adults in economically productive age groups which is thought to be a consequence of male overrepresentation in risk groups such as homeless, prisoners, seasonal migrant workers and people living with HIV among male homosexuals (ECDC and WHO, 2014). In the European Union/European Economic Area (EU/EEA) 26.8% of new cases are from foreign origin, ranging from 85% in Norway and Sweden to 15.6% in Portugal and 0.2% in Bulgaria and Romania (ECDC and WHO, 2014).

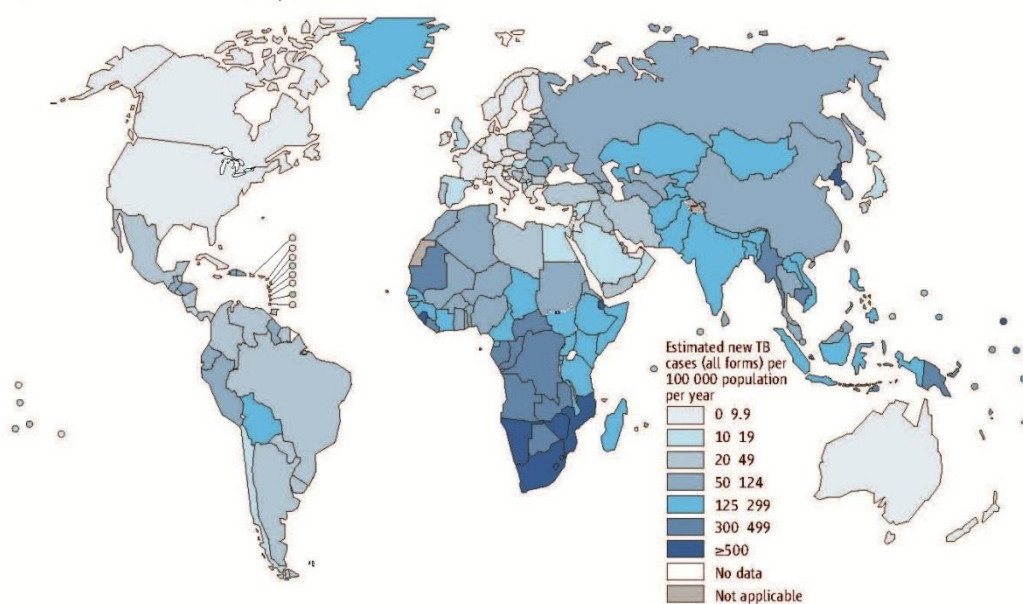


Figure 1.1. Estimated incidence rates of tuberculosis in 2012. Values per 100,000 individuals per year (WHO, 2012).

1.2. *Mycobacterium tuberculosis*

The genus *Mycobacteria* has recently been assigned to the new order of *Corynebacteriales* (Goodfellow, 2012). The majority of the 150 species that comprise this genus are non-pathogenic environmental bacteria that possess striking similarities with other bacteria of the phylum *Actinobacteria* such as *Streptomyces* (Scherr and Nguyen, 2009). Evolution within this genus has originated a few successful and widespread host-adapted pathogens such as *Mycobacterium leprea*, *M. avium*, and particularly those included in the *Mycobacterium tuberculosis* complex (MTC), the etiologic agents of TB. The species comprising this group share as high as 99.9% nucleotide similarity and identical 16S rRNA sequences (Böddinghaus et al., 1990; Sreevatsan et al., 1997) yet full sequencing of the ≈ 4 Mbps genome of *M. tuberculosis* (Cole et al., 1998) revealed several variable regions of differentiation enabling the distinction of the various species (Gordon et al., 1999). *Mycobacterium tuberculosis* (MTB), the major etiologic agent of human tuberculosis, has no known environmental reservoir and is now thought to have originated in Africa and co-evolved with its human host for more than 70,000 years (Comas et al., 2013). This indicates a very stable relationship between humans and these bacteria. In fact, some hypotheses suggest that early lineages of MTBC were highly prevalent in the human population suggesting that the infection would not be very detrimental (Comas et al., 2013), perhaps due to a lower virulence of those lineages, as can be observed today by studying differences in disease progression in different strains of MTB (de Jong et al., 2008). Recent evidence has shown that in some cases bacterial infection may induce protection against other microorganisms (Perry et al., 2010; Selbach et al., 2008); if so, it may represent an increase in evolutionary fitness in specific conditions such as those that occurred during the migration of humans out of Africa and consequent encounter of new pathogens. Moreover, when considering the co-evolution between MTB and humans we must not discard the impact that this microorganism might have had in the generation of adaptive changes in human genome (Barnes et al., 2011).

Mycobacterium tuberculosis are rod-shaped, non-spore-forming, aerobic bacteria typically measuring approximately 0.5 μm in diameter and 3 μm in

length, this last varying according with period of culture (Cook et al., 2009). They are slow growing bacteria with a doubling time of 12-24h under optimal conditions and are classified as acid-fast bacilli due to a unique cell wall structure (Knechel, 2009). The MTB peculiar cell wall is in fact one of its major features, providing a strong impermeable barrier to toxic compounds, whether being drugs or molecules released by the host immune system (Delogu et al., 2013). The mycobacterial cell membrane is constituted by an asymmetrical lipid bilayer and a periplasmic region of arabinogalactan-peptidoglycan polymer which constitutes the cell wall (Hoffmann et al., 2008; Zuber et al., 2008). The outer-membrane of mycobacteria is one of the thickest ($\approx 10\text{nm}$) reported in bacteria (Brennan and Nikaido, 1995; Engelhardt et al., 2002), representing one of the greatest obstacles to TB therapy. It is comprised of long fatty acids, designated mycolic acids in the inner leaflet and of glycolipids and waxy components on the outer layer. In the innermost side of the periplasmic space there is a thin layer of peptidoglycan covalently linked to arabinogalactan and lipoarabinomannan which in turn are bound to the mycolic acids on the outer-membrane (Figure 1.2). Interestingly, when Hoffman and colleagues (2008) engineered a mycolic acid-deficient *Corynebacterium glutamicum*, the bacteria were unable to produce an outer-membrane. They used *C. glutamicum* as a model since they are closely related to mycobacteria yet able to survive without the mycolic acids while such mutations are lethal in mycobacteria (Portevin et al., 2004). Overall, the mycobacterial membrane has a high hydrophobic nature, keeping hydrophilic compounds from entering the cytoplasm whereas the thickness of the membrane makes diffusion of hydrophobic compounds a slow and difficult process (Brennan and Nikaido, 1995). Furthermore, the membrane has a very low fluidity with a melting temperature above 70 °C (Liu et al., 1996).

Other surface structures that constitute major virulence factors in mycobacteria are secretion systems. So far, five type VII secretions systems have been described in mycobacteria (ESX1-5) (Abdallah et al., 2007), among which only three, ESX-1, ESX-3 and ESX-5 have known substrates and are classified as being virulence factors (Houben et al., 2014) These systems use the hydrolysis of ATP to drive the secretion of proteins and protein complexes across the membrane and cell wall of mycobacteria. ESX-1 is the most studied of these

secretion systems and its absence was identified as one major reason why the vaccine strain *M. bovis* BCG classified as attenuated (Lewis et al., 2003; Pym et al., 2002a). Amongst several substrates, the ESX-1 secretes two major culture filtrate proteins, ESAT-6 and CFP-10 (Stanley et al., 2003), now designated EsxA and EsxB. These two small proteins are highly immunogenic and are the basis of the Interferon- γ release assay (IGRA) (Diel et al., 2011; Mishra et al., 2010) which allows the distinction of MTB-infected individuals from those previously immunized with BCG, since the latter lacks ESX-1, EsxA and EsxB. For this reason the IGRA test is much more informative than the Mantoux intradermal injection test of purified protein derivate (PPD) of *M. tuberculosis* (Nayak and Acharjya, 2012).

ESX-3 is involved in zinc and iron homeostasis and is required for siderophore-mediated iron acquisition and growth of MTB in culture (Serafini et al., 2009, 2013; Siegrist et al., 2009, 2014). ESX-5 is also an essential virulence factor mediating the secretion of EsxN, and members of the PPE and PE_PGRS protein family (Bottai et al., 2012; Houben et al., 2012; Di Luca et al., 2012), which have been recently shown to trigger a range of innate immune responses, being also targets of the adaptive immune system (Balaji et al., 2007; Bansal et al., 2010; Ramakrishnan, 2000; Sampson, 2011). ESX-5 disruption has also been shown to lead to decreased cell wall integrity and increased susceptibility to detergents and hydrophilic antibiotics (Bottai et al., 2012).

A significant portion of the studies cited throughout this thesis, as well as in the work I present, involve the use of a model species of mycobacteria, *M. smegmatis* that is neither part of the MTC nor an intracellular pathogen. This bacterium is a saprophytic environmental microorganism which may cause opportunistic disease though most of the time easily controlled by the immune system. Many studies use these bacteria as models of MTB since there is significant genome conservation within the mycobacteria genus. Manipulation of MTB requires biosafety-level 3 facilities, highly trained personnel and carries a risk of accidental exposure. Moreover, being a fastidious microorganism, the long generation time of MTB makes experiments very time consuming. *M. smegmatis* is a BSL-1 microorganism with a generation time of 3 h. By using

this bacterium it is possible to draw significant insights about its physiology, metabolism, membrane composition and transport and also genetic engineering and expression of MTB proteins. In fact, many studies on the ESX secretion systems were performed in *M. smegmatis* prior to MTB (Coros et al., 2008; Siegrist et al., 2009). Also, dormancy genes regulated by the highly conserved Dos regulon were found to be present in both MTB and *M. smegmatis* (Lin et al., 2009). Concerning host response to infection, *M. smegmatis* are readily killed by macrophages and are susceptible to the microbicidal mechanisms of the host, such as nitric oxide release and phagosome acidification (Anes et al., 2006).

In this thesis, the use of *M. smegmatis* has served a differed purpose. Being part of the same genus as MTB but devoid of a clear pathogenic life cycle, *M. smegmatis* are perfect bacteria to compare with MTB at the level of immune response generated by the host and putative pathogen-dependent immune-modulatory mechanisms. While the same purpose could be achieved using other bacteria such as *Escherichia coli*, the greater phylogenetic distance could confuse interpretation of the result. For example, the presence of the pathogen associated molecular pattern (PAMP) LPS in *E. coli*, which is absent in mycobacteria, would be difficult to compare with MTB and to elucidate details of its virulence factors. When comparing MTB and *M. smegmatis* PAMPs, we can associate different phenotypes with subtle differences in PAMP molecular structure. Such is the case of the abundant MTB-cell wall lypoglycan, ManLam, which differs from that of *M. smegmatis* in the simple fact that this last isn't capped by mannose (Briken et al., 2004). So this comparison between MTB and *M. smegmatis* will ease our task of revealing MTB-specific virulence and resistance factors and pathogenic mechanisms.

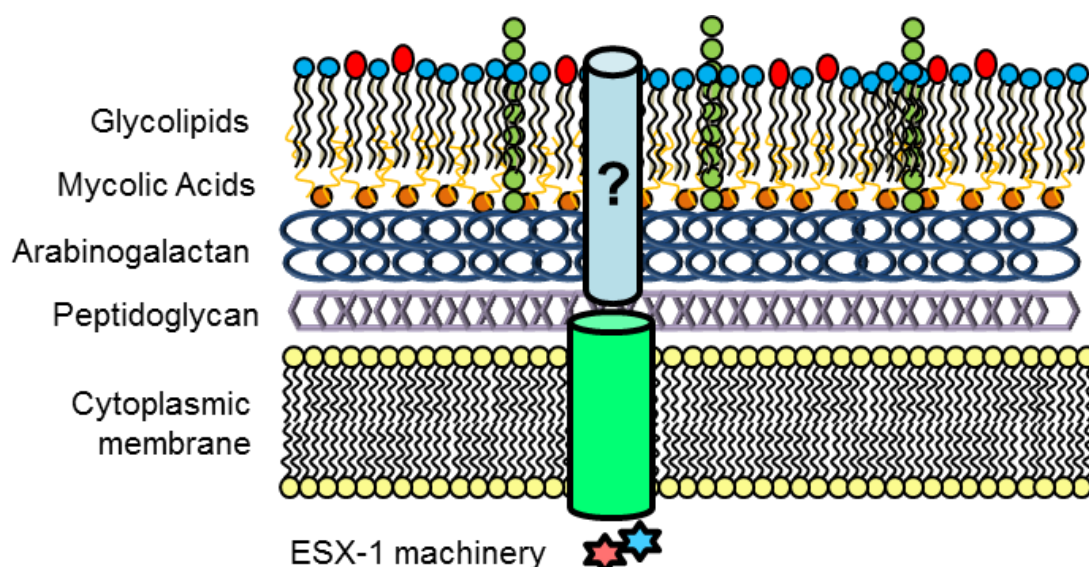


Figure 1.2. Model of the mycobacterial plasma membrane. Summarized representation of the several layers that comprise the mycobacterial plasma membrane as well as ESX-1 as an example of a transportation system. Interrogation point refers to the unknown intermediate channel protein in ESX other membrane transport.

1.3. Pathogenesis of tuberculosis

In most cases, the first infection with the bacilli occurs in the lungs (Figure 1.3) and may then expand to other organs. Mycobacteria enter the lung parenchyma and invade alveolar resident macrophages, dendritic cells, neutrophils and monocytes (Kang et al., 2011; Wallgren, 1948; Wolf et al., 2007). Recognition of MTB PAMPs through membrane and cytosolic receptors (see “MTB recognition by TLRs and NLRs”) will result in the expression of pro-inflammatory cytokines, chemokines and cellular adhesion receptors (Cooper, 2009; Cooper et al., 2011; Means et al., 1999). The signals initiated at this stage will result in activation and migration of innate immune cells such as monocyte-derived macrophages and dendritic cells (DCs) from the blood stream to the site of infection in the lung. Usually this recruitment results in the containment of the infection but in the case of MTB infection this might actually benefit the bacteria in the early stage of infection by providing additional cellular niches for the bacteria to invade and expand (Davis and Ramakrishnan, 2009). Nonetheless, this recruitment will also provide the basis for the initiation of adaptive immune

response by inducing the recruitment and maturation of DCs (Cooper, 2009). DCs that engulf bacteria mature and migrate to the lymph nodes (Bodnar and Serbina, 2001; Henderson et al., 1997; Hertz et al., 2001) where they will prime CD4 and CD8 T cells against mycobacterial antigens. There, DCs express on their surface MTB-derived peptides bound to HLA class I and II molecules. HLA class II molecules bind MTB exogenous peptides from the endosomal pathway (e.g. from the MTB containing phagosome). In contrast the HLA class I molecules that bind endogenous or foreign peptides from the cytosol (e.g. from cytosolic bacteria or viruses) followed by transport into the ER and then to the plasma membrane. Presentation through these two different classes of molecules will result in clonal expansion of different subsets of antigen-specific T cells. HLA class II-presented antigens will prime CD4 T cells whereas HLA class I-presented antigens will prime CD8 T cells (Parham, 2005). These primed T cells expand and migrate back to the focus of infection in the lungs.

Cellular immunity is crucial at this stage of infection and CD4 T cell Th1 immunity at the site of infection is especially critical for MTB control (Ernst, 2012; Flynn, 2004). The Th1 cytokines interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) are required for control of bacterial growth in both animals and humans. Mice deficient in these cytokines are unable to control MTB infection (Flynn et al., 1993; Mogues et al., 2001) and individuals with defects in the IFN- γ receptor were also shown to be highly susceptible to mycobacterial infections (Jouanguy et al., 1999). These cytokines promote the control of bacterial growth in the macrophage by activating intracellular killing mechanisms and partially overcoming MTB-induced phagosome maturation arrest (Jordao et al., 2008) (further described below in “Macrophages and Dendritic cells” and “Manipulation of host endocytic pathways by MTB”).

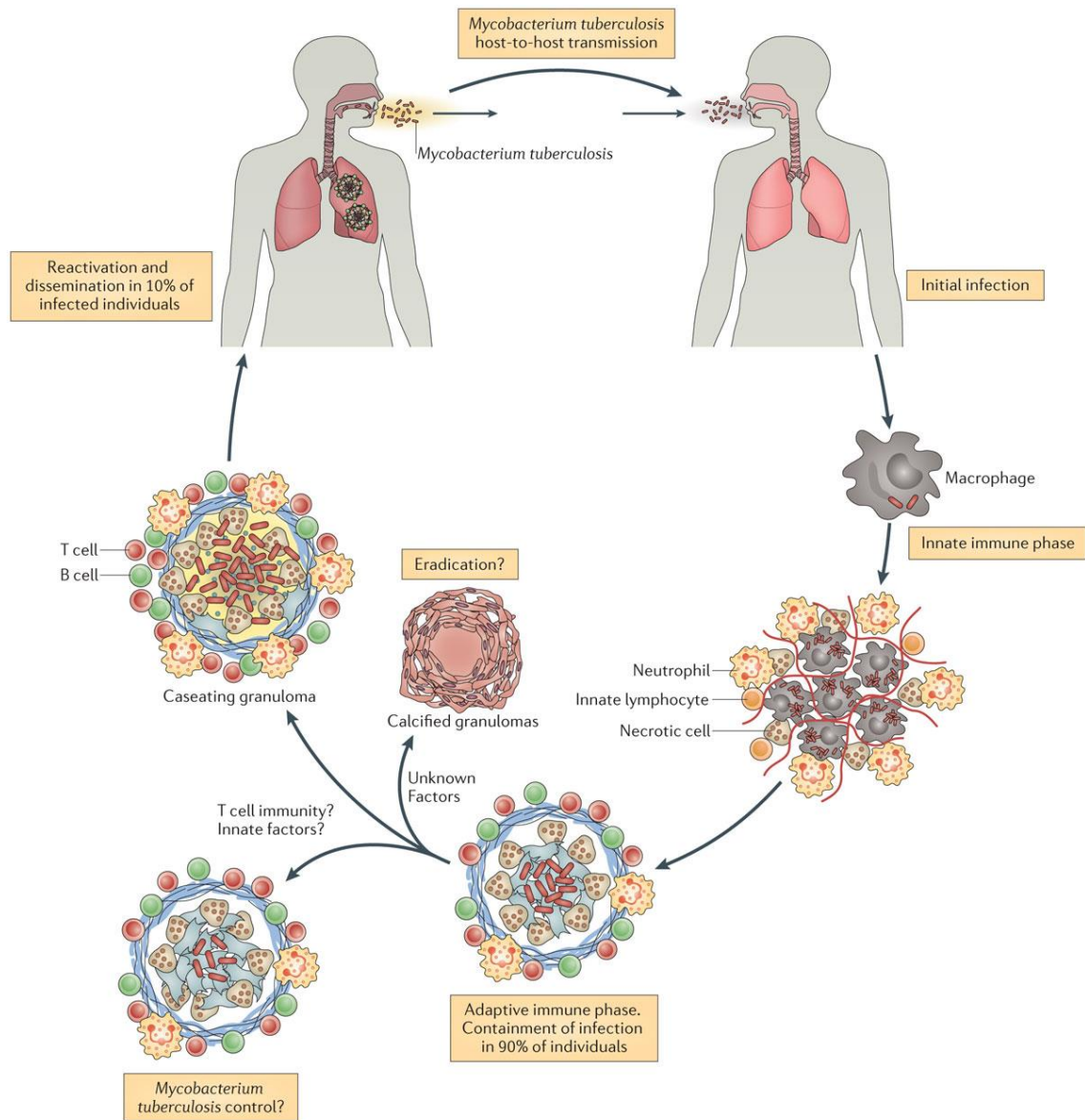
One of MTB's intriguing characteristics is its ability to attenuate those cellular responses mediated by CD4 and CD8 T cells, allowing an increase of the initial bacterial load in the lungs. One particular mechanism was proposed, involving the induction of massive proliferation of regulatory T cells upon direct presentation of MTB antigens by DCs (Shafiani et al., 2010) in the lung-draining lymph nodes, early after MTB infection. Regulatory T cells were shown to accumulate at sites of infection in both mice and humans (Kursar et al., 2007;

Scott-Browne et al., 2007) leading to a delayed entry of effector T cells into the lung (Shafiani et al., 2010). It is also interesting to note that during MTB infection there is a delay on the generation of antigen-specific immune response. Adaptive immune response is only measurable in humans after 42 days of exposure (Poulsen, 1950; Wallgren, 1948) and after 11-14 days in mice (Chackerian et al., 2002; Reiley et al., 2008; Wolf et al., 2008). In mice, the activation of MTB antigen-specific CD4 T cells first occurs in the lymph nodes draining the lungs and requires the transport there of live bacteria by DCs (Chackerian et al., 2002; Reiley et al., 2008). Interestingly this process takes 8 to 10 days during MTB infection of humans while during influenza virus infection it only takes 20 h (Ho et al., 2011). The reason for this delay is not yet clear. There is evidence that MTB inhibits the migration of DCs to the lymph nodes (Blomgran and Ernst, 2011).

Ultimately, equilibrium will be achieved between bacterial expansion and cellular immunity culminating in the formation of a granuloma – the hallmark of TB – and a state of latency. Granulomas formation is a chronic inflammatory response. Granulomas are formed at an early stage by a mass of macrophages and DCs and at the latter stage are joined by B and T cells. This standoff is probably the result of several described factors such as impaired MHC class II-mediated antigen presentation (Pancholi et al., 1993), down regulation of mycobacterial antigen expression (Bold et al., 2011; Egen et al., 2011), induction of regulatory T-cells (Scott-Browne et al., 2007) and resistance to IFN- γ -induced macrophage activation (Fortune et al., 2004; Ting et al., 1999). Remarkably, studies show that although there is stabilization in bacterial numbers during this chronic silent stage of infection, a subpopulation of bacteria remains actively replicating. Instead of a stasis, it may be that there is a dynamic immunologic equilibrium, demonstrated by evidence of bacteria replication in mice (Gill et al., 2009) and accumulation of mutations in non-human primates (Ford et al., 2011) during latency. Nevertheless bacterial gene regulation during latency has been described with particular emphasis on the bacterial regulon controlled by the two-component signal transduction system of mycobacteria, the DosR-DosS which is induced during hypoxia (Park et al., 2003), as well as by nitric oxide (Voskuil et al., 2003) and carbon monoxide

(Kumar et al., 2008). This system controls the expression of genes which allow the use of alternative energy sources (Muñoz-Elías and McKinney, 2005). Working against this system are “resuscitation proteins”, resembling the resuscitation-promoting factor (Rpf) of *Micrococcus luteus*, a secreted protein which enables bacteria to leave the nutrient-starved state (Chao and Rubin, 2010).

As previously referred, 5% to 10% of latently infected individuals will develop TB. This reactivation mostly occurs due to immune depression of the infected individual. HIV infection and consequent depletion of CD4 T cells is one the greatest causes of TB reactivation. Therapeutic inactivation of TNF- α is another major described cause of TB possibly due to a decrease in antimicrobial activity in macrophages (Clay et al., 2008). Another interesting possibility is a destabilization of the equilibrium inside the granuloma due to alterations in cell trafficking. Considering that this equilibrium is maintained by constant migration of fresh phagocytes and T cells to the granuloma, when chemokine deletion mutant mice were engineered, these showed a decreased ability to control the infection (Scott and Flynn, 2002). The result of reactivation will be the propagation of bacteria and extrusion from the granuloma. In the most infectious cases, individuals suffer extensive lung-tissue damage, thereby generating large open spaces filled with bacilli, a condition termed cavitary TB (Kaplan et al., 2003; Rodrigo et al., 1997). Curiously, individuals infected with HIV and with depleted CD4 T cells experience this phenomenon less frequently comparatively with individuals with a regular T cell population (Kwan and Ernst, 2011). In cavitary TB, the large cavities, connected to the airways provide an efficient pathway for bacteria expectoration and transmission.



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Figure 1.3. The pathogenesis of tuberculosis.
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1.4. Macrophages and Dendritic cells

Macrophages and DCs function as effector cells in the cellular immune response against TB forming large networks able to recognize the molecular patterns of invaders. Macrophages are professional phagocytes, a relatively straightforward definition, first given by Rabinovitch (Rabinovitch, 1967). Classic dendritic cells as first defined by Steinman and Cohn were clearly distinct from macrophages since they had little phagocytic ability and corresponded to the cells present in T cell areas in lymphoid organs and migratory cells in afferent lymph. Since then, this definition broadened to include phagocytic DCs and resulted in a division in numerous functional subsets of cross-presenting, migratory, myeloid, lymphoid, tolerogenic, and inflammatory or TipDCs. DCs are considered the antigen presenting cells par excellence, yet macrophages are also described to possess this ability leading to a division between those that include all these cells in the macrophage group or the DC group (Geissmann et al., 2010; Jenkins and Hume, 2014). A plethora of subpopulations of macrophages and DCs have been described, that are related to functional differences, as well as to their location in the body. In the lung, resident dendritic cells and alveolar macrophages are some of the first cells to respond to an invasion by foreign microorganisms. Infection will also lead to the recruitment of another population of monocyte-derived dendritic cells. The distinctions between these cells are less perceivable *in vivo* than those found in purified laboratory cultures. Although several markers have been proposed to distinct subsets of lung DCs and macrophages, the large overlap between these markers and different cell lineages as well as often subtle differences in their increased or decreased expression lead to an often mis-characterization of these populations and a misconception of their exact role during infection (Guilliams et al., 2013).

The populations of primary human cells analyzed in the following chapters of this thesis correspond to macrophages and DCs derived from a common circulating precursor, CD14 monocytes, and differentiated *in vitro*. For the purposes of this thesis I will apply a broad definition of macrophage and dendritic cell assuming that both cell types possess an inherent ability to phagocytize and to present antigens to T cells.

Macrophages play a crucial role in the inflammatory response and account for approximately 10% of the total cells of any organ (Hume, 2006). After reaching the infection site, they rapidly internalize the invading organism and kill it through a variety of processes that occur within the endocytic pathway such as the release of toxic nitrogen and oxygen species, acidification and lysosomal-mediated killing dependent on acidification of the phagosomal lumen and activation of lysosomal enzymes (Hope et al., 2004) (see “Cathepsins in the intracellular response against *M. tuberculosis*”). This antimicrobial activity is necessary not only to control the infection by killing the invading organism but also to process and present its antigens to T lymphocytes (see “Regulation of antigen presentation in MTB-infected cells”) in the context of major histocompatibility complex (MHC) molecules, as seen in mice (Hope et al., 2004) or their human counterpart, the human leukocyte antigens (HLA).

Macrophages have been classified as classically (M1) and alternatively (M2) activated macrophages. Prototypical M1 activation is mediated by IFN- γ and lipopolysaccharide (LPS). These macrophages mediate defense against bacteria, viruses and parasites with potent antimicrobial response and induction of IL-12-mediated Th1 response and secretion of pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6 (Guirado et al., 2013). As previously mentioned, IFN- γ -activated macrophages induce a potent release of reactive oxygen or nitrogen species that are able to destroy the bacterial invaders. Curiously this release will affect the phagosomal environment by increasing the pH and resulting in a lowered activity of low pH-dependent proteolytic enzymes. The consequence of these events will be partial proteolysis of the pathogen, with preservation of antigenic peptides to be presented to lymphocytes (Russell et al., 2009; Yates et al., 2007). This is the opposite of what occurs when no inflammatory stimulus is produced and macrophages silently eliminate the phagolysosomal contents due to high proteolytic activity and destruction of antigenic peptides (Russell et al., 2009).

M2 macrophages can be activated by stimuli with different cytokines, including IL-4 or IL-13 and have an anti-inflammatory role with induction of Th2 responses with secretion of IL-10 and TGF- β as well as reduced responsiveness to IFN- γ and decreased iNOS expression (Guirado et al., 2013;

Martinez et al., 2008). M2 macrophages participate in wound healing and resolution of inflammatory process. They rapidly clear phagocytized molecules and recent studies suggest they preserve much of the features of resting macrophages (M0), with rapid progression of phagosome maturation and lysosome fusion and highly acidic and proteolytic response (Balce et al., 2011; Canton et al., 2014). However, the M1 and M2 classification might be an oversimplification since these cells have been shown to retain the ability to switch from one state to another in response to changes in the environment (Mylonas et al., 2009; Stout et al., 2005). Moreover, often we can find features of both types of macrophages in alveolar macrophages. These have been shown to participate in pathogen clearance (M1) yet they do not trigger an exacerbated inflammatory response and actively suppress the production of pro-inflammatory cytokines (M2) (Martinez et al., 1997).

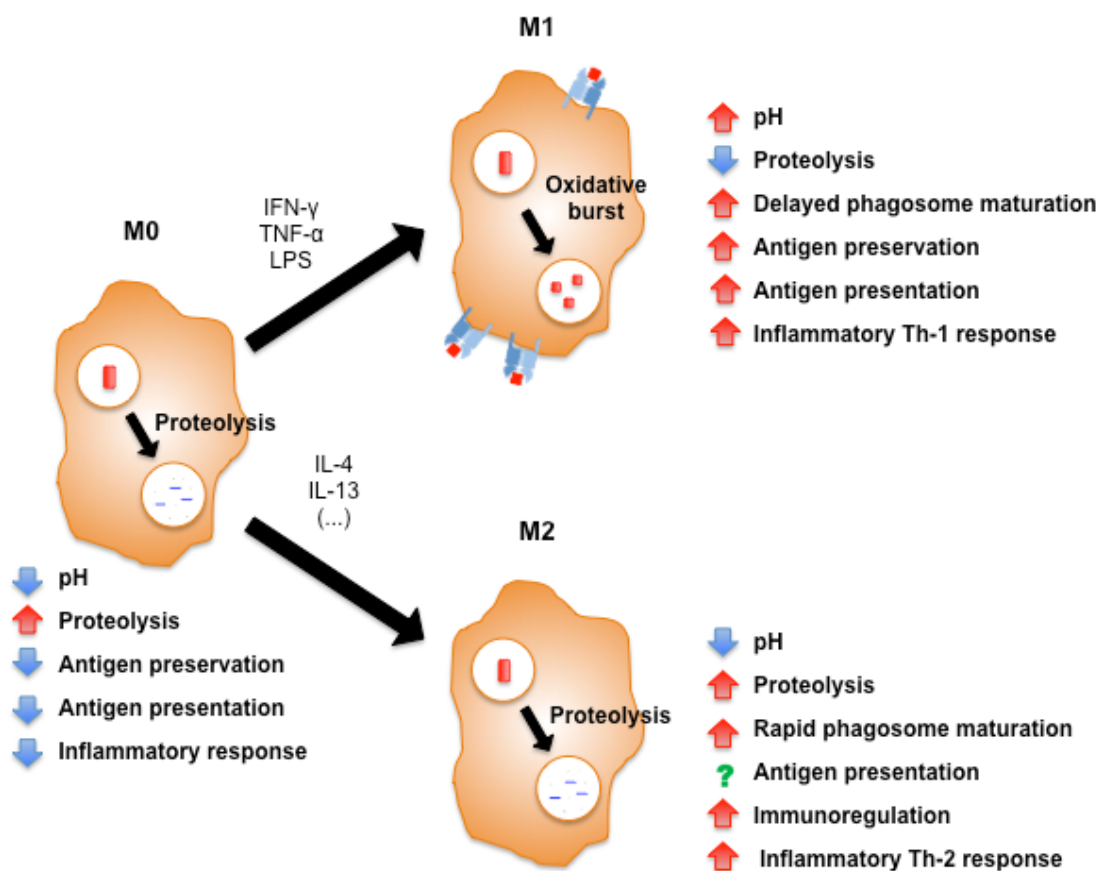


Figure 1.4. Macrophage polarization and its relevance for the endocytic/antigen presentation pathways

Dendritic cells are a minor population in most tissues, in contrast to the much more abundant resident macrophages. However, DCs are capable of forming large networks that spread to various parts of the body, such as the skin, intestine and the lungs (Hope et al., 2004). Their main purpose is to gather antigens that will be processed and presented to lymphocytes following migration of DCs into the lymphoid organs (Cella et al., 1997a). While performing their role in monitoring exposure of body surfaces to antigens, these cells are considered immature DCs (imDCs), that express low levels of MHC and co-stimulatory molecules but with a high capacity to phagocytose invading organisms as determined in mice models (Puré et al., 1990; Reis e Sousa et al., 1993). In the presence of tissue injury, inflammation or infection, imDCs are stimulated to migrate towards lymph nodes (Dudziak et al., 2007). There they mature and acquire the capacity to stimulate naïve T lymphocytes while at the same time losing their active phagocytic and endocytic functions (Banchereau et al., 2000). Indeed, DCs have developed specific control of endocytic functions in order to allow efficient antigen presentation (Mellman and Steinman, 2001). These cells contain lower levels of active lysosomal proteases and lysosome acidification is inefficient in the immature stage due to the limited recruitment of the proton pumping vATPase to endosomes and lysosomes, relative to macrophages (Delamarre et al., 2005; Russell, 2007; Trombetta et al., 2003). The mild pH of the endocytic pathway of DCs assures an efficient processing of pathogen antigens (Savina et al., 2006), in which cysteine cathepsins and their natural inhibitors cystatins play a crucial role (Kopitar-Jerala, 2006; Zavasnik-Bergant et al., 2005, 2006) (see "Regulation of antigen presentation in MTB-infected cells"). Since one of the most direct ways to control protease activity is by pH this can constitute an adaptation of these cells, but it also can favor bacterial persistence in a less degradative environment.

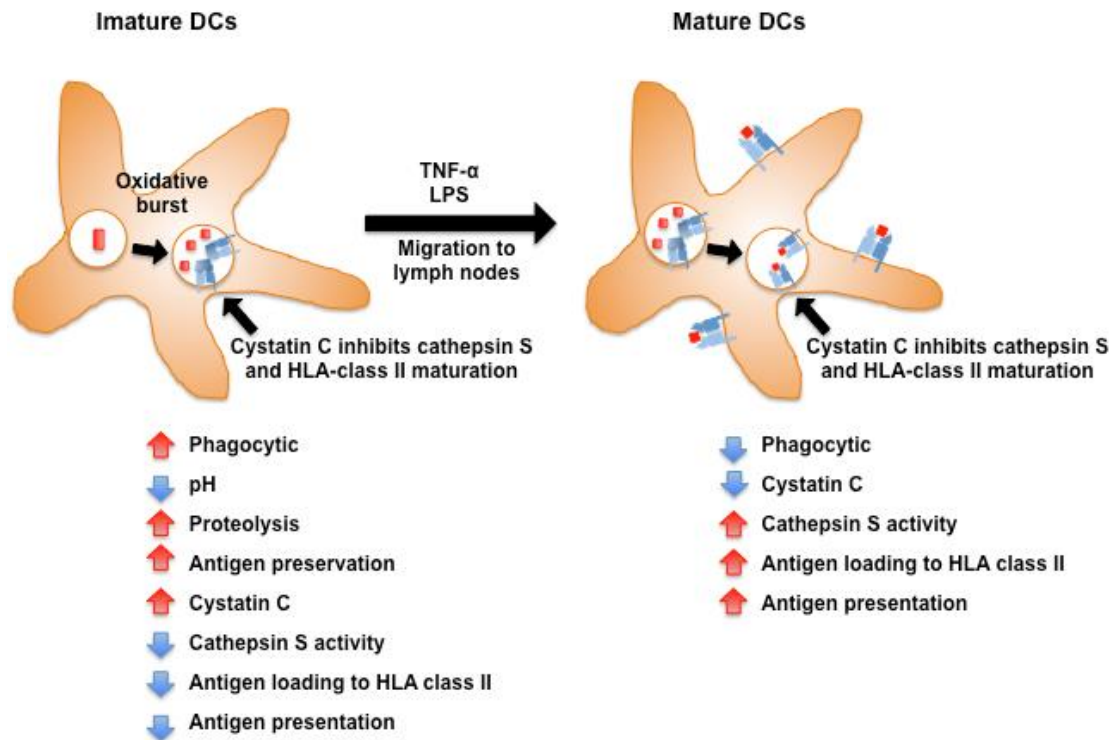


Figure 1.5. Dendritic cells maturation its relevance for the endocytic and antigen presentation pathways

1.5. MTB uptake by Macrophages and DCs

The first step in the phagocytic process is the internalization of the foreign body or pathogen. Several surface molecules of MTB have been implied in receptor-mediated uptake of the bacteria by phagocytes and most evidences come from studies in macrophages (Schäfer et al., 2009). As mentioned before in this thesis, the ability of MTB to infect phagocytes appears to be important for its dissemination so it is not hard to imagine the variety of intermediates that have evolved for this process. However, this multitude of molecules involved creates a challenge for researchers studying the contributions of single molecules on MTB uptake; in the end we must keep in mind that all these processes are occurring together and that in the same cell the bacteria might face different fates which will in some occasions lead to its survival, others to its death but all contributing to the global pathogenesis of TB.

Bacteria are recognized by opsonic and non-opsonic receptors. In the former, bacteria are coated with various host proteins such as antibodies, complement

proteins, lectins or surfactants; all these molecules serve to target the bacteria for recognition by macrophage receptors of host proteins. In contrast, pattern recognition receptors (PRRs) directly recognize bacterial ligands on their surface. In the initial stages of infection, non-opsonic binding or surfactant-mediated opsonic binding is thought to occur more prominently since there is limited serum and complement proteins in the alveoli (Schluger, 2001; Torrelles et al., 2008). In the latter stages of infection, with the buildup of adaptive immunity, the contribution of opsonic receptors might help to contain the infection (Stokes et al., 1993, 1998).

Regarding uptake, the non-opsonic receptors described include c-type lectins, integrins and scavenger receptors. C-type lectins play an important role in mycobacterial binding and in generation of inflammatory responses due to the presence of carbohydrate rich molecules in the mycobacterial surface (Schäfer et al., 2009). This group includes the macrophage mannose receptor (MMR), DC-specific intracellular adhesion molecule 3–grabbing non-integrin (DC-SIGN or CD209), and Dectin-1. The MMR is the predominant C-type lectin expressed on non-activated human macrophages and is expressed in alveolar macrophages (McGreal et al., 2005), monocyte-derived macrophages and dendritic cells (Stahl and Ezekowitz, 1998), but not on monocytes (Stahl, 1992). MMR was the first C-type lectin to be associated with recognition of MTB, binding to its mannosylated surface structures (Torrelles and Schlesinger, 2010). It also has high avidity and affinity for endogenous mannans serving to maintain homeostasis of the host (Lee et al., 2002). DC-SIGN also recognizes mannosylated cell wall components and is present on immature and mature DCs and small sets of macrophages (Bleijs et al., 2001; McGreal et al., 2005). Although it is not expressed on non-activated human macrophages, its expression can be up-regulated upon MTB infection (Tailleux et al., 2005). DC-SIGN mediates the internalization and uptake of bound particles and directs them to mature phagolysosomes (Philips and Ernst, 2012). Both MMR and DC-SIGN bind to the abundant MTB lipoglycan ManLAM. ManLAM is mannosylated in pathogenic mycobacteria whereas it binds to the arabinose capped- or inositol phosphate capped-LAM of environmental mycobacteria such as (Briken et al., 2004). ManLAM has been investigated as a potential virulence factor and

was shown to inhibit the TLR-2-mediated inflammatory effects of LPS, LPS-induced TNF- α secretion and IL-12 p40 mRNA synthesis probably through ligation to mannose receptors (Knutson et al., 1998) of macrophages. In DCs, DC-SIGN binding by LAM was shown to partially inhibit LPS-induced DC maturation and induce IL-10 secretion (Geijtenbeek et al., 2003). However, mice lacking the MMR are equivalent to wild-type controls in terms of their ability to control MTB (Court et al., 2010).

Concerning DC-SIGN, inhibition studies revealed that DC-SIGN is the major mycobacterial receptor in human DCs (Tailleux et al., 2002). Studying DC-SIGN in mice has been proven difficult due to the existence of 7 paralogs of DC-SIGN in mice (Ehlers, 2010). Nonetheless, engineering of BCG and *M. marinum* mutants that do not add mannose caps to LAM showed that the absence of ManLAM does not lead to attenuation of infection in macrophages or in mice (Appelmelk et al., 2008). DC-SIGN also recognizes other mannosylated components such as hexamannosylated phosphatidylinositol mannan (PIM6). Nevertheless, mutants defective in both ManLAM and PIM6 showed no observable phenotype (Driessen et al., 2009), suggesting that other ligands in the mycobacterial cell wall may engage DC-SIGN and other receptors.

Dectin-1 is expressed by macrophages, DCs, monocytes, and a subset of T cells (Taylor et al., 2002). It binds to mycobacteria and β -glucan (absent in MTB), but its MTB ligand is still unknown. However, functional studies show that Dectin-1 ligation triggers phagocytosis and intracellular signaling cascades, including synergistic interactions with the TLRs, leading to a pro-inflammatory response (Lee and Kim, 2007) and oxidative burst (Zhang et al., 2000).

The complement C3 receptors (CR1, CR3 and CR4) play an important role in both opsonic and non-opsonic phagocytosis of MTB by macrophages (Ferguson et al., 2004; Schlesinger, 1993; Schlesinger et al., 1990). They interact with surface polysaccharides (Cywes et al., 1996), PIMs and glycopeptidolipids (Villeneuve et al., 2005). CR3 (also named integrin $\alpha_M\beta_2$ or CD11b/CD18 or Mac-1) mediates MTB phagocytosis yet uptake via this receptor leads to unclear host responses. Although bacterial uptake is decreased, the intracellular bacterial growth is kept unchanged (Melo et al.,

2000). It was also shown that CR3-mediated phagocytosis does not induce the release of reactive oxygen species (Le Cabec et al., 2000). However, CR3-knockout mice failed to reduce bacterial burden or lessen pathological lesions when MTB was injected intravenously (Hu et al., 2000).

Opsonic Fcγ receptors are also important receptors in macrophages and monocytes and are relatively high expressed in alveolar macrophages. However, since these receptors require an opsonizing antibody for their activity they do not play a role in the initial phagocytosis of MTB (Schlesinger et al., 1990). However, when the bacteria are opsonized with MTB-specific antibodies and activate these receptors, there is enhanced phagosome–lysosome fusion (Armstrong and Hart, 1975), facilitating an increased host macrophage protective response.

Pulmonary collectins (soluble C-type lectins) play an important role in MTB interaction with alveolar macrophages (Beharka et al., 2002; Crowther et al., 2004; Henning et al., 2008; Kuronuma et al., 2004). Upon entry into the alveoli, MTB encounters surfactant, and two surfactant-associated collectins, surfactant protein A (SP-A) and surfactant protein D (SP-D). SP-A has been shown to enhance PRR activity, increase phagocytosis, alter production of pro-inflammatory cytokines, and decrease reactive nitrogen and oxygen species in response to stimuli (Crowther et al., 2004; Wright, 2005). In contrast, SP-D decreases phagocytosis of MTB by binding to the mannose caps of ManLAM and inhibiting the interaction with the MMR (Ferguson et al., 2006) whereas SP-D-opsonized MTB display reduced intracellular growth in macrophages due in part to increased phagosome–lysosome fusion (Ferguson et al., 1999, 2006).

1.6. MTB recognition by TLRs and NLRs

The understanding of the mechanisms of innate immune recognition was greatly enhanced by the findings of Medzhitov and Janeway more than 15 years ago (Medzhitov and Janeway, 1998), when they proposed a key role for the Toll protein as regulators of immune signaling. Since then, mammalian toll-like receptors (TLRs) have been shown to sense various PAMPs at the cell

surface or within phagosomes (Lee and Kim, 2007). Therefore, TLRs recognize extracellular or membrane encased foreign organisms. More recently, another family of PRRs was described, the NOD (nucleotide binding and oligomerization domain)-like receptors (NLRs) (Inohara et al., 2005; Meylan and Tschopp, 2006; Meylan et al., 2006). These differ from TLRs by being soluble proteins that survey the cytoplasm. Moreover, some members of the NLR protein family have been shown to form cytosolic complexes with pro-caspase-1 leading to its activation and consequent processing of pro-IL-1 β and thereby revealing yet another route for the engagement of the inflammatory process. These complexes were termed “inflammasomes” (Martinon et al., 2002) and since then many different studies have identified different types of inflammasomes that assemble in response to different stimuli. These findings gave new perspectives on the study of intracellular pathogens and raised new questions about the possible interplays between the membrane-attached TLRs and this cytosolic family of receptors. In fact, many reports show synergistic crosstalk between TLR agonists and NOD2 agonists in pro- and anti-inflammatory cytokine release (van Heel et al., 2005; Netea et al., 2005; Uehara et al., 2005; Watanabe et al., 2004, 2006), adding to the difficulty in discriminating their role in innate immunity.

TLRs are a highly conserved family of trans-membrane receptors comprised by 12 members in mammals and responding to a variety of ligands (Akira et al., 2006; Brightbill et al., 1999; Medzhitov et al., 1997; Rock et al., 1998; Trinchieri and Sher, 2007). They are expressed in a multitude of cells, including macrophages, neutrophils, lymphocytes, and DCs, as well as by alveolar epithelial cells (Armstrong et al., 2004; Dasari et al., 2005; Hayashi et al., 2003; Kadowaki et al., 2001; Krutzik and Modlin, 2004). They possess an extracellular amino-terminal leucine-rich repeat (LRR) domain that recognizes PAMPs and an intracellular carboxy-terminal tail that is homologous to the interleukin 1 receptor (IL-1R) (Basu and Fenton, 2004). The receptor contains a Toll-IL-1R (TIR) domain that forms a scaffold for the assembly of signaling intermediates. TLRs are either expressed on the cell surface such as TLR2 and TLR4, or intracellularly such as TLR8 and TLR9. MTB and its cell wall components are recognized by several TLRs, including TLR1, TLR2, TLR4, TLR6, and TLR9 (Jo

et al., 2007). Mycobacterial cell wall PIMs, lipomannan (LM) and the 19-kDa lipoprotein have been shown to interact with TLR2 (Dao et al., 2004; López et al., 2003; Vergne et al., 2004). After specific ligand binding, TLRs such as TLR2 and TLR4 initiate an intracellular signaling cascade, which generally leads to differential activation of NF- κ B and an inflammatory response (Akira et al., 2006; Malhotra et al., 1990; Poltorak et al., 1998). TLRs can be regulated by the pulmonary surfactant by means of increasing the expression of a negative regulator, interleukin-1 receptor-associated kinase M (IRAK-M) and IL-10 production in macrophages (Nguyen et al., 2012). SP-A also up-regulates the surface expression of TLR2 on human macrophages, while inhibiting the intracellular signaling of TLR2 and TLR4, which results in a dampened pro-inflammatory response (Henning et al., 2008). Moreover, TLRs have also been increasingly found to interact with other surface receptors leading to a modulation of the inflammatory response (Cambi et al., 2005; Kawai and Akira, 2011).

NLR family members also play a major role in innate immunity, being a second line of recognition molecules inside macrophages and mediating cell death pathways (Inohara et al., 2005). NLRs are mainly expressed in antigen presenting cells, including macrophages, DCs and epithelial cells. NOD1 and NOD2 are part of the NLR family (Ting et al., 2008). NOD1 is found in multiple cell types, whereas NOD2 protein expression is abundant in human macrophages (Brooks et al., 2011; Ogura et al., 2001). NOD1 and NOD2 recognize specific muropeptides found in the peptidoglycan layer of Gram-positive and Gram-negative bacteria (Inohara et al., 2003). NOD2 recognizes an N-glycolylated form of muramyl dipeptide (GMDP) found in MTB and controls the nature of the inflammatory response and subsequent fate of MTB and *M. bovis* BCG in human macrophages (Brooks et al., 2011). Upon ligation, NODs indirectly induce NF- κ B and AP-1 activation by signaling through mitogen-associated protein kinase (MAPK), ultimately leading to the production of pro-inflammatory cytokines (Chen et al., 2004). NOD2 can also directly bind and activate caspase-1 and interact with the NLRP1/NLRP3 inflammasomes (Hsu et al., 2008). Mice studies have revealed that although NOD2 does not appear to have a significant role in the control of early MTB infection, it may be important

at latter stages (Divangahi et al., 2008; Gandotra et al., 2007). Furthermore, in those studies MTB infection was shown to induce a decrease in pro-inflammatory cytokine production in NOD2-knockout macrophages but without affecting intracellular growth of the bacteria.

As mentioned before, inflammasomes are multiprotein complexes. They share caspase-1 as a terminal effector that cleaves pro-IL-1 β (Schroder and Tschopp, 2010) yet they are part of different NLR subfamilies with different sensor domains. NLRP3 mediates activation of caspase-1 in response to MTB (Mishra et al., 2010) yet NLRP3-deficient mice display no increased susceptibility to *M. tuberculosis* infection (Schroder and Tschopp, 2010). In contrast, mice lacking the adaptor protein PYCARD/ASC, which links several distinct NLRs to inflammasome activation, are highly susceptible to MTB infection and exhibit early mortality, high bacterial burdens, and poorly formed granulomas (Schroder and Tschopp, 2010).

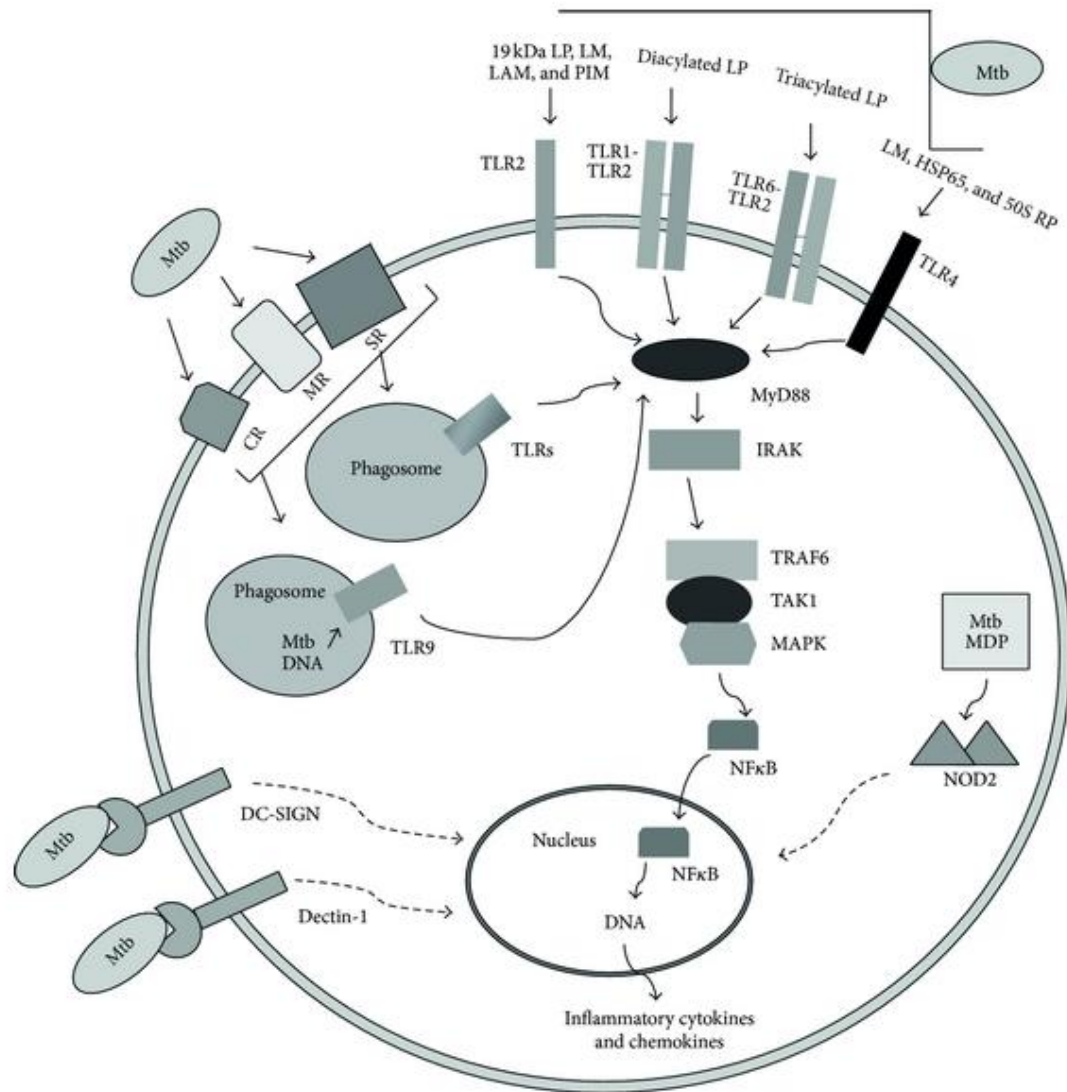


Figure 1.6 Immune recognition of MTB during phagocytosis.

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1.7. Manipulation of host endocytic pathways by MTB

There is now considerable evidence supporting the thesis that MTB subverts intracellular immune mechanisms by modulating cellular trafficking and phagosomal maturation, consequently avoiding lysosomal-mediated killing, to generate a favorable niche for its growth (Blomgran et al., 2012; Chackerian et al., 2002; Khader et al., 2006). Then, MTB induces its release from the host cell through virulence factors such as ESX-1, inducing necrotic cell death and promoting the recruitment of fresh phagocytes to be infected and thus allow the growth and expansion of MTB population (Davis and Ramakrishnan, 2009). Yet,

so far I have presented several studies that show different outcomes and responses to MTB infection related to the type of cell, its activation and also the receptors triggered. It is reasonable to assume that in the midst of all this immunomodulation MTB is likely to occupy different intracellular niches throughout the endocytic/phagocytic pathways (Figure 1.8), which may lead to phagosomal arrested maturation or to phagolysosome fusion; to the initiation of the autophagic process, or the cell death by necrosis or apoptosis. The different outcomes of these pathways will dictate the survivability of the bacilli and also the availability of its antigens to be presented by the antigen presentation machinery. I will now address these pathways, how they are exploited by MTB and how the host cell responds.

Although the intracellular pathways of mycobacterial survival are well characterized in macrophages, less information is available on the intracellular fate of MTB in other host cells, especially in myeloid DCs, which harbor MTB in humans and mice (Wolf et al., 2007). The transcriptional profile of MTB is significantly different when the bacteria are inside DCs as compared with macrophages (Tailleux et al., 2008), which indicates that comparative studies of the intracellular trafficking and fate of MTB in macrophages and DCs are likely to yield valuable information about the life cycle of the pathogen.

The regular phagocytic process involves the engulfment of the bacteria through actin-mediated membrane movements with the sequential recruitment of Rab GTPases to the phagosomal membrane, which will then recruit vacuolar ATPases, leading to the acidification of the phagosomal lumen. The mature acidic phagosome will then fuse with a lysosome and merge their contents, leading to the acquisition and activation of acidic hydrolases (Guirado et al., 2013). Bacteria enclosed in the phagosomal compartment must suffer attrition in an iron-limiting, carbon-poor, hypoxic, nitrosative and oxidative environment (Schnappinger et al., 2003). MTB is able to resist this bactericidal response by synthesizing antioxidants, repairing DNA and proteins, maintaining intracellular pH and also cell wall integrity (Boshoff et al., 2003; Buchmeier et al., 2000; Colangeli et al., 2009; Darwin and Nathan, 2005; Master et al., 2002; Vandal et al., 2008, 2009). Furthermore, as previously mentioned, MTB may alternate its metabolism by activating the dormancy regulon thus enabling its survival in an

hypoxic and growth-limiting environment (Griffin et al., 2011; Leistikow et al., 2010; Marrero et al., 2010; Ryndak et al., 2010).

The best documented and repeatedly mentioned feature of MTB-infected phagocytes is the alteration of phagosomal trafficking and phagosomal maturation arrest. The first report of these events comes from the observation that MTB does not colocalize with ferritin-labeled lysosomes (Armstrong and Hart, 1971). Since then, this arrested phagosome has been characterized by a variety of molecular markers. The MTB-phagosome is an early endosome-like compartment, retaining early endosomal markers that are usually cleared from maturing phagosomes (Ferrari et al., 1999; Kyei et al., 2006; Roberts et al., 2006). It remains in communication with early recycling endosomes, thereby acquiring iron from the transferrin/transferrin receptor system (Clemens and Horwitz, 1996; Sturgill-Koszycki et al., 1996); and also acquiring host cholesterol and fatty acids to be used as sources of carbon. There is a failure to replace the loss of the early marker Rab5 with late marker Rab7 on the mycobacterial phagosome (Sun et al., 2007; Via et al., 1997), and numerous late endosomal or lysosomal markers are diminished or absent, including the vacuolar ATPase (vATPase), that results in a failure to fully acidify (Russell, 2001). MTB resides in a unique phagosome with an abnormally high pH of approximately 6.2 and with limited fusion with lysosomes (Deretic et al., 2006; Russell, 2001) favoring its survival and growth. Nonetheless, even when MTB is coated with immune serum, resulting in uptake via Fc receptors and delivery to lysosomes, the bacteria are able to resist the acidic environment and grow (Armstrong and Hart, 1975).

1.7.1. MTB lipids and proteins in phagosomal maturation arrest

Compared with phagosomes containing latex beads or heat-killed pathogenic mycobacteria, phagosomes containing live pathogenic mycobacteria exhibit diminished phosphatidylinositol-3-phosphate (PI3P) (Chua and Deretic, 2004; Purdy et al., 2005; Vergne et al., 2005). PI3P is a critical lipid intermediate in the recruitment of the vacuolar GTPases to the phagosome (Kaufmann and Britton, 2008) and is generated by a class III phosphatidylinositol 3-kinase (PI3K). The

exact mechanism by which MTB impairs PI3P generation is not fully clear. Reports claim that ManLAM can inhibit normal calcium increase in the cytosol, causing a disruption in calmodulin complex formation with the PI3K (Chua et al., 2004; Deretic et al., 2006; Fratti et al., 2003) and thereby prevent the recruitment of PI3P to the phagosomal membrane (Vergne et al., 2003). MTB can also inhibit calcium signaling by inhibiting sphingosine kinase (Malik et al., 2003). Yet another mechanism proposed for PI3P degradation occurs by a PI3P phosphatase secreted by MTB, SapM, which was shown to block phagosome fusion with late endosomes (Vergne et al., 2005). However, a BCG mutant lacking SapM displayed no defect in its ability to arrest phagosome maturation (Festjens et al., 2011). Due to the lack of PI3P in the phagosomal membrane, PIP3 effectors, such as early endosomal autoantigen 1 (EEA1) (Fratti et al., 2001; Schlesinger et al., 2008) and hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), are diminished on phagosomal membranes (Connolly and Kusner, 2007; Kusner, 2005; Russell, 2001). Lack of PI3P and Hrs may interfere with recruitment of the endosomal sorting complex required for transport (ESCRT) that restricts the intracellular growth of mycobacteria (Philips et al., 2008). Also, EEA1 and Syntaxin-6 are required for the delivery of lysosomal hydrolases, cathepsins, and vATPases (Guirado et al., 2013).

Another important lipid in MTB-mediated modulation of the endocytic pathways is trehalose dimycolate (TDM). Extraction of MTB surface lipids results in increased localization of MTB in more acidified compartments and diminished intracellular survival, a phenotype which can be partially reversed by purified TDM (Indrigo et al., 2003). Consistent with these results is the evidence that mutants defective in TDM generation depict altered phagosome maturation (Katti et al., 2008) and latex beads coated with TDM exhibit delayed phagosome maturation (Axelrod et al., 2008; Indrigo et al., 2003).

Besides the previously mention SapM, other MTB proteins play a role in phagosome maturation arrest, such as PtpA (tyrosine phosphatase), PknG (serine/threonine kinase), LpdC (lipoamide dehydrogenase), Zmp1 (Zn²⁺ metalloprotease) and GAP (GTPase-activating protein) (Philips, 2008).

PtpA is required to arrest phagosome maturation and enhances MTB survival in human macrophages (Bach et al., 2008) probably acting together with other bacterial effectors to prevent acquisition of activated Rab7 on the phagosome. Latex beads coated with PtpA reduce phagolysosomal fusion. The mechanism proposed for PtpA involves its cytosolic substrate Vps33B, which is part of a family of proteins that aid SNARE assembly (soluble N-ethylmaleimide-sensitive fusion protein attachment receptors) to promote vesicle docking and fusion. Consistently, Vps33B depletion by RNAi dampens the delivery of lysosomes to latex bead-containing phagosomes (Bach et al., 2008).

PknG is also required for phagosome maturation arrest, and *M. smegmatis* expressing it are able to prevent phagosome-lysosome fusion (Walburger et al., 2004), although the mechanism is still unknown.

LpdC role in phagosome maturation was discovered because LpdC from MTB and BCG, but not *M. smegmatis*, exhibits a cholesterol-dependent interaction with coronin 1 in infected macrophages. Coronin 1 is recruited and retained at the phagosomal membrane, where it activates calcium–calcineurin signaling. Activated calcineurin blocks the delivery of MTB to lysosomes, probably by dephosphorylating an unknown host cell substrate (Pieters et al., 2013). This facilitates the long-term survival of MTB within phagosomes. When *M. smegmatis* are made to express the MTB LpdC, the resulting phagosome retains coronin 1 with enhanced bacterial survival (Deghmane et al., 2007).

ESX-1 secretion appears to be important for phagosomal arrest in both MTB and *M. marinum*, since mutants defective in the ESX-1 secretion system exhibit reduced arresting of phagosome maturation (MacGurn and Cox, 2007; Tan et al., 2006). However this secretion system is absent in the BCG genome and yet this species is still capable of mediating phagosome-lysosome fusion arrest. In MTB, known substrates of the ESX-1 system (EsxA, EsxB and EspA) appeared dispensable, suggesting an unidentified effector (MacGurn and Cox, 2007). Interestingly, the ESX-1 machinery could lead to the exact opposite effect since it was shown that this system is an important inflammasome activator (Koo et al., 2008; Master et al., 2008; Mishra et al., 2010). The mechanism by which the inflammasome enhances phagosome maturation is unknown, but Zmp1, a zinc-

dependent metalloprotease of MTB, antagonizes its activity, thereby inhibiting phagosome maturation (Master et al., 2008).

The ESX-1 secretion system of pathogenic mycobacteria has been implicated in phagosomal membrane damage leading to the leakage of bacterial antigens or even whole bacterial escape to the cytosol. This phenomenon was especially known to occur in *M. marinum* (Gao et al., 2004; Smith et al., 2008) yet In human macrophages and DCs, ESX-1 also appears to promote the escape of MTB from the phagosome (van der Wel et al., 2007). The two secreted effectors of ESX-1, EsxA (ESAT-6) and EsxB (CFP-10) were implicated in membrane damage yet EsxA alone appears to be sufficient to mediate the process (Hsu et al., 2003; de Jonge et al., 2007a; Smith et al., 2008). Also, damage to the phagosome may activate autophagic clearance of the bacteria although no direct evidence has been reported. An interesting consequence of membrane damage and leakage would be the increased accessibility of bacterial antigens to the class I antigen presentation machinery and also of MTB PAMPs to cytoplasmic receptors. In fact, cell wall N-glycolyl-muramyl dipeptide is only able to stimulate NOD2 in bacteria carrying an effective ESX-1 system (Coulombe et al., 2009; Pandey et al., 2009; Stanley et al., 2007). Similarly, as previously mention, ESX-1 is required for the bacteria to activate the inflammasome in the host cytoplasm (Koo et al., 2008; Mishra et al., 2010). In addition to facilitate access of antigenic peptides the ESX-1 is also directly involved in generation of adaptive immunity since both EsxA and EsxB are prominent antigens recognized by both CD4 and CD8 T cells (Brodin et al., 2004).

1.7.2. The role of Autophagy in MTB-infected cells

Autophagy is an essential cellular homeostasis mechanism through which protein aggregates, damaged organelles or also intracellular microbes are encased in double membrane vacuoles termed autophagosomes which undergo maturation and fusion with lysosomes (Levine et al., 2011; Shintani and Klionsky, 2004). The complex membrane dynamics of autophagosome formation are not completely understood. Autophagy is induced by nutrient starvation, stress inputs, and immune mediators through the inhibition of

mammalian target of rapamycin (mTOR) leading to the recruitment of PI3K and consequentially to the generation of PI3P (see “MTB lipids and proteins in phagosomal maturation arrest”) (Levine et al., 2011). Autophagosome formation depends upon two protein-protein and protein-lipid conjugation systems (Atg5/Atg12 and Atg8/LC3). This process is not only responsible for removing in excess or damaged organelles but also to provide means of cell survival during starvation through turnover of macromolecules (Shintani and Klionsky, 2004). Autophagy sequesters bacteria that access the host cell cytosol, which can promote both bacterial eradication and antigen presentation. The cytosolic bacteria are coated with ubiquitin, recognized by adaptor proteins and targeted to the autophagic machinery to be enclosed in a double-membrane compartment that matures to an autolysosome. It's noteworthy that bacteria adapted to the cytosol such as *Listeria* and *Shigella* inhibit autophagy. When *M. marinum* escapes the phagosome it becomes ubiquitinated and is sequestered into structures resembling autophagosomes. Still this process appears not to require LC3 or Atg5, so it might be an independent pathway (Collins et al., 2009). As previously mentioned, MTB also appears to escape to the cytosol yet since most autophagy studies were performed with BCG (which lacks ESX-1), it is still unclear if MTB is attacked by autophagy, or whether it inhibits it, or if it is sequestered in an independent pathway such as what occurs with *M. marinum*. Still, it is worth mentioning that an RNAi screen in human monocytic cell line THP-1 revealed that endogenous negative regulators of autophagy are required for MTB growth (Kumar et al., 2010).

Basal autophagy does not appear to make a major contribution to bacterial clearance, but autophagy is bactericidal when activated by multiple stimuli, including starvation, rapamycin, IFN- γ , vitamin D, TLR4/TLR7 (Delgado et al., 2008; Gutierrez et al., 2004; Harris et al., 2007; Xu et al., 2007; Yuk et al., 2009). When autolysosomes fuse with MTB-containing phagosomes they deliver peptide-ubiquitin conjugates with anti-mycobacterial activity (Alonso et al., 2007; Ponpuak et al., 2010). Of great interest was the fact that this process was shown to overcome MTB inhibition of PI3P and to decrease bacillary viability by reinstating phagosomal maturation (Gutierrez et al., 2004). Indeed the stimulation of autophagy increased phagosomal co-localization with Beclin-

1, a subunit of the PI3K complex, as well as with LC3. The finding that autophagy can be a means to improve bacterial killing and surpass MTB phagosomal maturation arrest opened the door for a mechanism to improve the presentation of MTB antigens. Making use of rapamycin as an autophagy inducer, another group showed that by increasing autophagic activity, both macrophages and DCs exhibited an increase in presentation of antigen 85 (Jagannath et al., 2009). Mice immunized with rapamycin-treated DCs infected with BCG showed enhanced Th1 CD4 cell-mediated protection when challenged with MTB.

1.7.3. Necrosis vs Apoptosis in MTB persistence in host cells

Cell death is a host defensive mechanism in response to many stimuli, including microbial infection. However, intracellular pathogens often manipulate the cell death pathways, inhibiting them in order to persist and replicate in the host and then induce them later to enhance their dissemination. So far, a plethora of cell death types have been described, including apoptosis, autophagy, mitoptosis, necrosis, necroptosis, netosis, oncosis, pyroptosis, and pyronecrosis (Parandhaman and Narayanan, 2014). Concerning TB, there is increasing evidence associating the virulence of specific strains with their ability to inhibit apoptosis and ultimately direct the host cell towards a necrotic cell death (Behar et al., 2010). Apoptosis is a process mediated by a caspase cascade that results in the formation of apoptotic vesicles. These vesicles are then degraded by adjacent macrophages in an anti-inflammatory fashion (Harris and Keane, 2010) although there is evidence that apoptosis may also lead to a pro-inflammatory response dependent on MTB-infected apoptotic neutrophils (Persson et al., 2008). Apoptosis also promotes antigen presentation through cross-presentation of MTB antigens to CD8 T cells leading to the development of an adaptive immune response (Behar et al., 2011; Schaible et al., 2003; Winau et al., 2006) (see “Cross-presentation in the generation of MTB-specific immunity”). Unlike apoptosis, necrosis leads to a pro-inflammatory response due to the permeabilization of the cellular membrane and leakage of the cell contents. Inside the granuloma, the characteristic caseous area is caused by

necrotic cell death of macrophages infected with a high burden of bacteria and the release of inflammatory cytokines by infected macrophages and uninfected leukocytes that surround the granuloma (Repasy et al., 2013). *In vitro* studies in macrophages have also shown that apoptotic cells release fewer bacteria compared to necrotic cells (Molloy et al., 1994). Several MTB mutants that promote macrophage apoptosis were shown to be attenuated *in vivo* (Hinchey et al., 2007; Velmurugan et al., 2007). The avirulent strain of MTB, H37Ra, for example, stimulates the production of the pro-apoptotic prostaglandin E2 (PGE2) while the virulent strain H37Rv induces the production of lipoxin A4 (LXA4) which induces macrophage apoptosis (Chen et al., 2008). Mice lacking an upstream gene, 5-lipoxygenase, required to produce LXA4 are also more resistant to the infection (Divangahi et al., 2010). Two other types of cell death, pyroptosis and pyronecrosis may also occur during MTB infection, through the activation of the inflammasome. As previously mentioned, MTB activates the inflammasome in an EsxA (ESAT-6) dependent fashion (Mishra et al., 2010) but there is no direct evidence that it induces pyroptosis and pyronecrosis.

1.7.4. Activators of the mycobactericidal mechanisms of macrophages

I previous mentioned that activation by specific cytokine mediated stimulus induces changes in the macrophage intracellular response to foreign bodies (see “Macrophages and Dendritic cells”). While MTB is able to survive and replicate in resting macrophages, MTB killing activity is enhanced when a stimulus is provided by other macrophages or T cells, with the release of IFN- γ , TNF- α and vitamin D. IFN- γ was shown to be essential for MTB control in humans and mice (Chan et al., 1992; Jouanguy et al., 1999). Primed T cells release IFN- γ and activate macrophage killing mechanisms by increasing the expression of nitric oxide synthase 2 (NOS2) and production of reactive nitrogen species (RNS) (Chan et al., 1992). Nonetheless, there is still much debate on the actual contribution of RNS to MTB killing in human macrophages. As mentioned above, IFN- γ also stimulates autophagy-dependent delivery of lysosomes to the MTB-containing phagosome. The curious finding is that IFN- γ impairs the rate of phagosome-lysosome fusion in latex bead-containing

phagosomes, causing diminished hydrolytic and proteolytic activity in the phagosome (Trost et al., 2009; Yates et al., 2007). Although it is still unknown if this delay also occurs in pathogen-associated phagocytosis, it might be beneficial to the host, by preserving intact peptides to be presented to T cells by HLA class II molecules.

TNF- α is also essential for control of MTB in humans and in animals (Solovic et al., 2010) by activating macrophages for killing intracellular MTB and to modulate apoptosis of infected cells (Balcewicz-Sablinska et al., 1998; Clay et al., 2008). TNF- α can also act with IFN- γ to activate macrophages for the control of MTB growth. Nevertheless, excessive TNF- α response contributes to the immunopathology of TB (Bekker et al., 2000; Tobin et al., 2010), resulting in tissue damage. MTB has developed mechanisms to modulate TNF- α production through the secretion of a bacterial adenylate cyclase (Agarwal et al., 2009), thereby enhancing virulence and lung inflammation in mice.

Another relevant mechanism that has been studied with interest is vitamin D-induced killing of MTB. The expression of the vitamin D receptor is dependent on vitamin D active form, 1,25- dihydroxyvitamin D₃ (calcitriol). This receptor mediates the transcription of the gene encoding cathelicidin, the precursor of LL-37, which was shown to kill MTB and other bacteria (Liu et al., 2007; Martineau et al., 2007). TLR2-mediated activation of human macrophages was also shown to increase the expression of the vitamin D receptor and cathelicidin (Liu et al., 2006) in the presence of calcitriol. Similarly to IFN- γ , calcitriol may activate autophagy since calcitriol-dependent expression of cathelicidin elicits the transcription of autophagy-related genes. Moreover, LL-37 is also recruited to the autophagosome (Yuk et al., 2009). Recent reports also implicate vitamin D in alterations in MTB lipid metabolism due to down-regulation of the pro-adipogenic peroxisome proliferator-activated receptor γ (PPAR γ) in infected macrophages (Salamon et al., 2014).

Besides these inducers of macrophage bactericidal response, some focus has been given to lipid-mediated inhibition of MTB growth. A study reported that inhibition of mycobacterial growth in macrophages could be observed after addition of several bioactive lipids such as arachidonic acid, sphingomyelin,

sphingosine, or ceramide (Anes et al., 2003). These effects were proposed to occur via enhanced actin nucleation on the phagosomal membrane. While it is not proven that these events are actin-dependent, previous studies have shown that MTB may modify the cytoskeleton of infected macrophages, modify actin filament network and inhibit the movements of lysosomes in the vicinity of newly formed phagosomes (Guérin and de Chastellier, 2000; Hart et al., 1987). This was shown to inhibit proper localization of the inducible nitric oxide synthase (iNOS), the enzyme responsible of nitric oxide synthesis, in the vicinity of the MTB-phagosome in mice (Miller et al., 2004). Purified phagosomes containing latex beads or non-pathogenic mycobacteria promote actin nucleation *in vitro* while phagosomes containing pathogenic mycobacteria were shown to inhibit this process (Anes et al., 2003). Stimulation of actin-dependent mechanisms, as was shown by addition of lipids such as arachidonic acid (Anes et al., 2003), might prove to be a pathway for improving MTB clearance in humans.

1.7.5. Regulation of antigen presentation in MTB-infected cells

In order to mount an effective immune response against MTB, the immunologically naïve cell populations that mediate adaptive immunity, such as T cells, need to be primed by cells carrying MTB antigens. This will lead to an antigen specific reaction that will target MTB and MTB-infected cells that display bacterial antigens. Foreign pathogen-derived peptides are expressed at the surface of APCs, like dendritic cells and macrophages, through binding to HLA class I and HLA class II molecules. HLA class II molecules bind exogenous peptides from the endosomal pathway (e.g. from the MTB containing phagosome) contrary to HLA class I molecules that bind peptides from the cytosol (e.g. from cytosolic bacteria or virus) upon transport to the ER. Presentation through these two different molecules will result in clonal expansion of different subsets of antigen specific T cells. HLA class II-presented antigens will prime CD4 T cells whereas HLA class I-presented antigens will prime CD8 T cells.

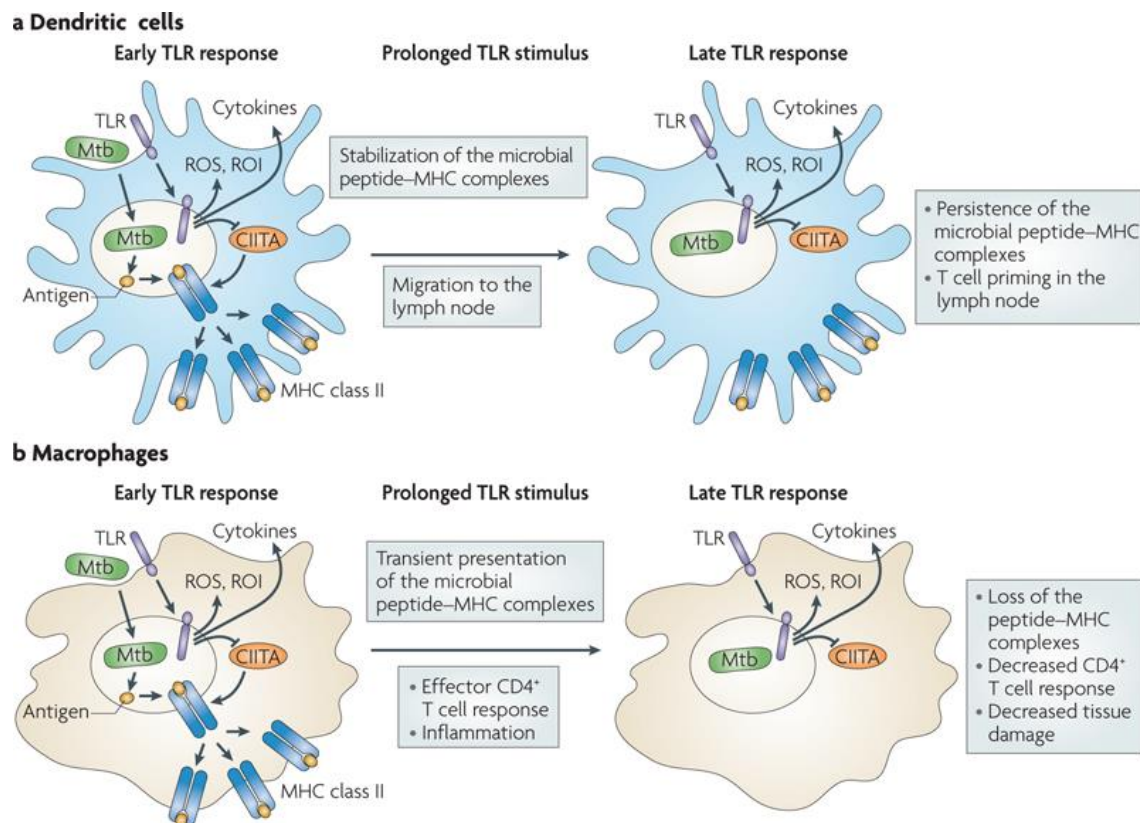
For the presentation of MTB antigens to occur, bacterial peptides must be produced by proteolysis and then bound to HLA class II molecules. HLA class II

molecules are assembled in the endoplasmic reticulum where they are also associated with a invariant chain (Ii) molecule to prevent premature peptide loading prior to delivery to the phagolysosome. In the phagolysosome, the invariant chain is degraded by proteases, particularly cathepsin S, leaving the class II-associated Ii peptide (CLIP) in the peptide-binding groove. Only then HLA-DM will catalyse the dissociation of CLIP and exchange with the phagolysosomal peptides (self or foreign). The peptide-HLA class II complex is then transported to the plasma membrane where it will interact with CD4 T cells (Wearsch and Cresswell, 2009). HLA class II-mediated antigen presentation can be negatively regulated by another HLA molecule, HLA-DO which binds to HLA-DM and inhibits its function, preventing CLIP dissociation (Wearsch and Cresswell, 2009). Another described mechanism occurs through transcriptional control of HLA-class II by the class II transactivator (CIITA) (Boss and Jensen, 2003; Harton and Ting, 2000; Masternak et al., 2000; Reith and Boss, 2008; Reith and Mach, 2001; Steimle et al., 1993, 1994). CIITA interacts in the assembly and action of several transcription factors, resulting in the induction of HLA class II transcription and also regulation of the invariant chain and HLA-DM (Boss and Jensen, 2003; Chang and Flavell, 1995; Chang et al., 1996). Control of CIITA expression is performed by different promoters in macrophages and DCs (Muhlethaler-Mottet et al., 1997). In macrophages the majority of these promoters are responsive to IFN- γ , leading to the induction of CIITA and HLA class II genes, while in immature DCs, the promoter was shown to induce CIITA independently of IFN- γ (Pai et al., 2002).

So far, there is considerable evidence that MTB interferes with the synthesis and expression of HLA class II molecules (Harding and Boom, 2010). Some studies propose that this regulation occurs directly in HLA class II gene promoters (Wang et al., 2005) while others propose that this regulation is mediated by the inhibition of CIITA (Kincaid and Ernst, 2003; Pai et al., 2003; Pennini et al., 2006, 2007; Wojciechowski et al., 1999). MTB-infected macrophages exhibit an inhibition of HLA class II molecules following approximately 15-24 hours after infection (Noss et al., 2000, 2001; Pai et al., 2003). A proposed model (Harding and Boom, 2010) implies TLR signaling in the decreased expression of HLA class II molecules via mitogen-activated

protein kinase (MAPK) activation, nitric oxide and the induction of inhibitory transcription factors that will lead to decreased expression of CIITA. The consequent decay of MHC class II molecules transiting through the ER and Golgi and delivery to the phagolysosome would take several hours, possibly explaining the delay in HLA class II inhibition mentioned above (Harding and Boom, 2010).

Assessing inhibition of HLA class II antigen presentation beyond the regulation of gene expression presents a more difficult challenge since it requires a functional analysis rather than a simple measure of expression. MTB was shown to inhibit the expression of several genes involved in antigen processing and presentation in macrophages via its 19 kDa lipoprotein, after prolonged TLR signaling (Pai et al., 2004). Mice studies revealed that high-frequency MTB infection of lung DCs resulted in a decreased ability to prime CD4 T cells without altering the surface expression of HLA class II molecules (Wolf et al., 2007). MTB was also shown to inhibit surface expression of HLA class II molecules through intracellular sequestration of these molecules (Hmama et al., 1998). Phagosome maturation arrest might also impact antigen presentation, although several studies indicate that this does not impair the processing of MTB antigens (Majlessi et al., 2007; Ramachandra et al., 2001, 2005). An interesting theory relates the impairment of HLA class II-mediated antigen presentation in macrophages to the need to reduce the pro-inflammatory response elicited by CD4 T cells in the infected tissues. Since macrophages mostly present antigens in the locus of infection, this mechanism would prevent tissue damage after prolonged exposure of macrophage receptors, such as TLRs, to MTB PAMPs. Meanwhile, DCs would require an enhanced activation by those receptors to increase the expression of HLA class II molecules while travelling to the lymphoid organs to prime CD4 T cells (Harding and Boom, 2010).



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Figure 1.7. The different antigen presentation responses of macrophages and dendritic cells to Toll-like receptor signaling.

(a) In DCs, TLR signaling induces maturation, which involves increased expression of peptide–MHC class II molecule complexes and co-stimulatory molecules. DC maturation also involves migration to lymph nodes, where DCs present antigen to naive T cells, priming the T cell response to pathogens. (b) By contrast, macrophages initially show little decrease in MHC class II antigen presentation and then, after prolonged stimulation (approximately 24 hours or more), show inhibition of antigen presentation with decreased expression of MHC class II molecules. This contrast in MHC class II levels results from regulation of the post-translational stability of MHC class II molecules, which is greatly enhanced during DC maturation but is not increased in macrophages upon TLR stimulation. Accordingly, TLR stimulation of DCs by microorganisms results in a final burst of antigen processing and the accumulation of a kinetic cohort of peptide–MHC class II molecule complexes that include microbial peptides; this cohort is expressed for a prolonged period and provides effective stimulation of naive T cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Harding and Boom, 2010), copyright 2010.

1.7.6. Cross-presentation in the generation of MTB-specific immunity

In spite of being encased by the phagosomal vacuole, MTB-infected cells are able to prime CD8 T cell through the HLA class I machinery. Accessibility of MTB antigens to the HLA class I machinery is thought to occur via cross-presentation (Guermonprez et al., 2002; Heath et al., 2004). Cross-presentation refers to the activation of CD8 T cells by APCs that obtained those antigens from a donor cell. This process involves an antigenic transfer between the cell initially infected and the antigen-presenting cell (Winau et al., 2005). For example, the antigenic content of apoptotic vesicles from macrophages are able to prime APCs for cross-presentation (Winau et al., 2005). Another type of cross-presentation was also found to take place through a direct pathway, which does not need an intermediary cell. There is no consensus about how this occurs, yet a proposed explanation states that antigens phagocytised by DCs and some macrophages are only partially degraded in the phagosome. Then they are transported into the cytosol where they are further broken down (Watts, 2006). Finally, upon being taken by the ER, they are loaded onto MHC class I molecules to be presented at cell surface to CD8 T cells.

In fact, as previously mentioned there are reports proposing that MTB is capable of escaping the phagosome and/or compromise its structural integrity through the Esx-1 secretion system. This event would permit the leakage of MTB antigens into the cytosol, thus rendering them accessible to the HLA class I-mediated antigen presentation pathway. So far, evidence of phagosomal escape has been shown to occur only in late periods of infection (days) while HLA class I-mediated antigen presentation occurs in hours (Grotzke et al., 2009, 2010). Studies also show that the ESX-1 machinery is not required to onset CD 8 T cell immunity (Woodworth et al., 2008), arguing that phagosomal escape might not explain cross-presentation. Still, antigen leakage into the cytosol remains an interesting hypothesis since proteins secreted by MTB are over-represented as antigens recognized by CD8 T cells (Woodworth and Behar, 2006). Generation of apoptotic vesicles is a pathway for cross-presentation of MTB antigens and was found to be important to provide protection against MTB in mice. Researchers found that DCs had a major role in crosspriming by taking up MTB antigens from apoptotic vesicles and then

migrating into the lymph nodes to prime CD 8 T cells (Winau et al., 2006). This was further confirmed by abrogating the expression of MHC class I molecules on the surface of the apoptotic cell, indicating that an intermediary DC was necessary.

Another pathway for cross-presentation might involve a non-apoptotic type of vesicle, the exosome. Exosomes are 50–150 nm membrane vesicles originating from multivesicular bodies by inward budding of endosomal membranes and are released via fusion of the limiting membrane of multivesicular bodies with the plasma membrane, resulting in release of intraluminal vesicles as exosomes (Schorey and Bhatnagar, 2008). Exosomes may play a role in both innate and adaptive immunity. They carry several MTB PAMPs, such as LAM, PIM, TDM and phenolic glycolipids (Beatty et al., 2000). Exosomes carrying these molecules can stimulate bystander cells and induce inducible nitric oxide synthase and secretion of pro-inflammatory cytokines such as TNF- α and RANTES (Bhatnagar and Schorey, 2007; Bhatnagar et al., 2007). Moreover, lipoprotein 19 kDa was also shown to be important for pro-inflammatory stimulation by exosomes (Stewart et al., 2005). Exosomes were already found to be successful antigen presenters in cancer immunotherapy. In the case of MTB, exosomes from BCG-infected macrophages were shown to stimulate CD4 T cells and CD8 T cells *in vitro* and *in vivo* (Giri and Schorey, 2008). Exosomes from macrophages treated with MTB culture filtrate proteins were found to induce antigen-specific IFN- γ and IL-2-expressing CD4 and CD8 T cells. When mice were vaccinated with these exosomes they exhibited similar Th1 response.

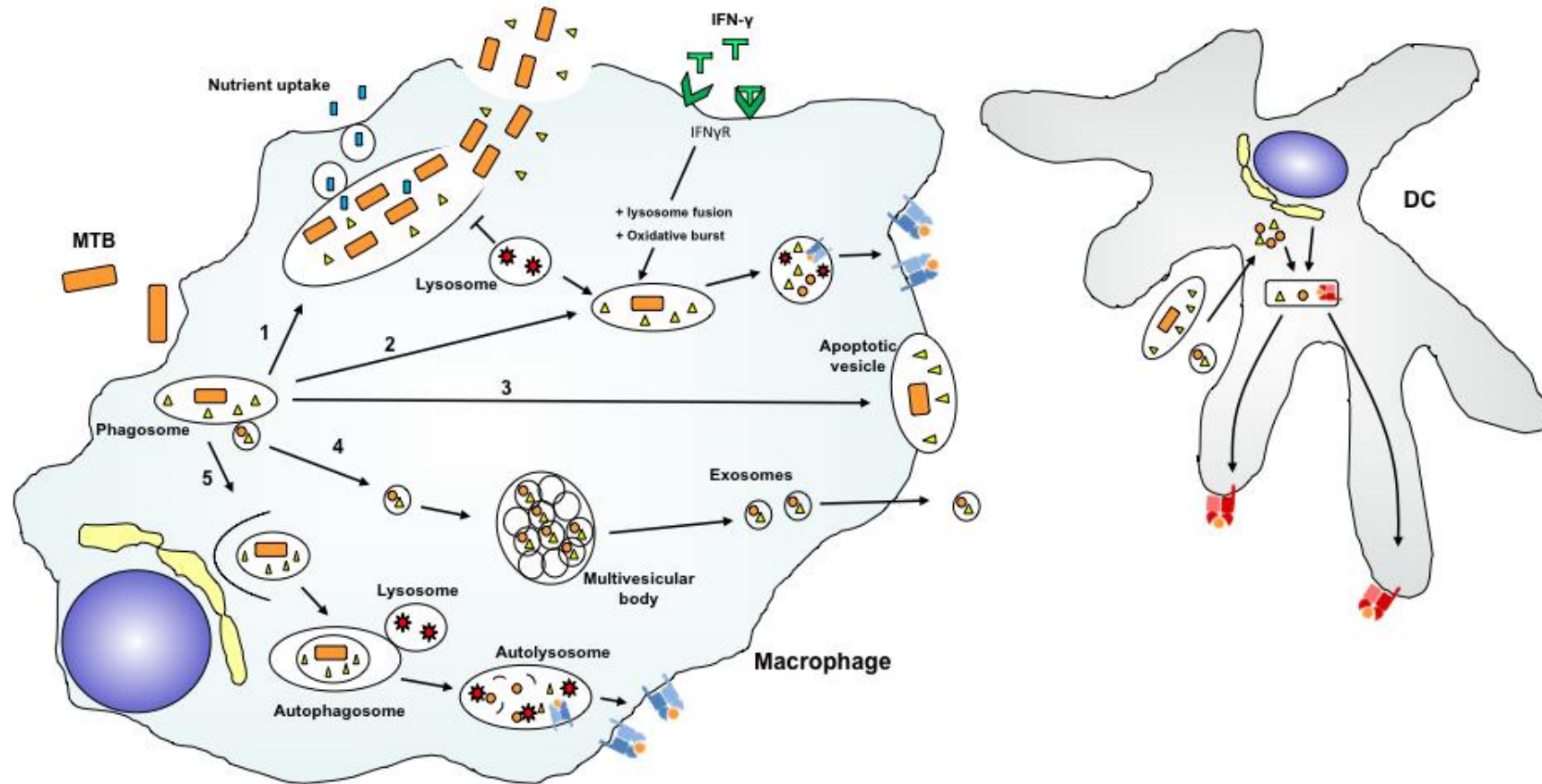


Figure 1.8. The endocytic/antigen presentation pathways during MTB infection.

(1) MTB impairs phagosome-lysosome fusion, replicates and causes necrosis of the cell. (2) External stimuli, such as IFN-γ released from T cells, induces a strong oxidative burst and phagolysosome-fusion leading to the destruction of the pathogen and presentation of its antigens via HLA class II. (3) Apoptosis induced with budding of the membrane and release of apoptotic vesicles with MTB and/or MTB antigens. (4) Multivesicular body formation leads to secretion of antigen-containing exosomes. (5) Formation of autophagosomes with fusion with lysosomes and consequent MTB elimination and antigen presentation. DCs cross-present MTB antigens by uptake of the contents of apoptotic vesicles and exosomes then present them via HLA class I.

1.8. Cathepsins in the intracellular response against *M. tuberculosis*

In 1955, Christian de Duve (Nobel Prize, 1974) discovered a distinct population of vesicles containing acidic hydrolases, among them a cathepsin protease, now known as aspartic cathepsin D. These vesicles were called lysosomes due to their abundance in hydrolytic enzymes (Christian de Duve, B.C. Pressman, R. Gianetto, R. Wattiaux, 1955). The finding that these hydrolases act on different substrates suggested that the lysosomes, also known as “suicide bags”, might play a role in intracellular digestion at acidic pH. Lysosomes were then discovered in several cell types, playing a role in intracellular acidic digestion and found to be involved in the degradation of extracellular material taken up via endocytosis and also intracellular material sequestered via autophagy (de Duve, 1983, 2005). For an efficient degradation of the internalized macromolecules, lysosomes possess a variety of hydrolytic enzymes including proteases, amylases, lipases and nucleases. Amongst the most abundant, widespread and studied proteases are aspartic, serine and cysteine cathepsins, as named according to the amino acid residue present at their catalytic site. Cathepsins include the serine proteases cathepsins A and G, the aspartic proteases cathepsins D and E and the cysteine proteases cathepsins B, C, F, H, K, L, O, S, V, X and W. Optimal activity of lysosomal cathepsins typically require a slightly acidic and reducing environment and most of them are unstable at neutral pH and suffer irreversible unfolding (Turk et al., 1995, 1999). Cathepsin S is an exception, being stable at neutral or slightly alkaline pH and retaining its activity (Kirschke et al., 1989). Cathepsin L is at the opposite extreme, being the most unstable cathepsin at neutral pH (Turk et al., 1993). Cathepsin B also shows irreversible loss of activity at pH accompanied by structural changes (Turk et al., 1994).

Different roles of cathepsins might be attributed to their substrates but also to their location. Most human cathepsins, such as cathepsins B, H, L, C, X, F and O, are ubiquitously expressed in human cells, and are probably involved in normal protein turnover. Others, such as cathepsins K, S and W are restricted to specific tissues, indicating a more specific role. Cathepsin K is highly

expressed in osteoclasts (Salminen-Mankonen et al., 2007) and is essential for extracellular matrix degradation and bone resorption. Cathepsin W is predominantly expressed in CD8 T cells and natural killer (NK) cells (Linnevers et al., 1997; Wex et al., 2003).

Cathepsin S is predominantly expressed in APCs, such as DCs and also in B-cells (Hsing and Rudensky, 2005). Cathepsin V (also named L2) is highly homologous to cathepsin L yet is only expressed in the thymus and testis (Brömme et al., 1999; Santamaría et al., 1998). Cathepsins are also known to act in other cellular compartments outside the scope of this thesis. Nonetheless it is worth mentioning that several cathepsins are secreted in order to cleave extracellular-matrix components such as elastins, collagens and proteoglycans as exemplified by cathepsins K, L, S, F, H, V and B (Brömme et al., 1996; Fonović and Turk, 2014a; Lutgens et al., 2007; Yasuda et al., 2004). Cysteine cathepsins exhibit broad specificity, cleaving their substrates preferentially after basic or hydrophobic residues, consistent with their roles in intracellular protein degradation (Turk et al., 2000). Most cathepsins are endopeptidases, whereas cathepsins X and C are exopeptidases. In addition to their endopeptidase activity, cathepsins B and H also exhibit exopeptidase activity (Zavasnik-Bergant et al., 2006).

Cathepsins are regulated by endogenous protein inhibitors, cystatins, thyropins and serpins, among others. These compete for the active site of the cathepsin and bind in a reversible fashion, preventing substrate binding. These regulators act in emergency situations, when cathepsins are leaked outside of their proper compartments or when pathogens release their own proteases, and inactivate them to avoid cell damage. They also act by regulating the activity of cathepsins by inhibiting them in the absence of their target or modulating their activity according to cellular responses (Klotz et al., 2011; Turk et al., 2002). For example, in immature DCs, HLA class II-invariant chain complexes accumulate and need to be processed by cathepsin S. This event will depend on the ratio between cathepsin S and its inhibitor cystatin C (Pierre and Mellman, 1998). Upon maturation, cystatin C is down-regulated, and the activity of cathepsin S increases, promoting degradation of the invariant chain and allowing the export of peptide-loaded class II molecules to the cell surface.

Cathepsins have been implicated in cell death pathways. Pyronecrosis, a process initiated by inflammasome activation is dependent on cathepsin B and is observable in shigellosis (Carneiro et al., 2009; Willingham et al., 2007). In addition, cathepsin D activation during pneumococcal infection was shown to induce apoptosis of macrophages, therefore promoting the death of this pathogen (Bewley et al., 2011). Another study reported that an MTB protein, Rv3364c, binds to cathepsin G on the plasma membrane of phagocytic cells and inhibits caspase-1 activity and pyroptosis (Danelishvili et al., 2011).

Several cathepsins have been found to play a role in antigen presentation. Cathepsins S, L, F and V were shown to degrade the invariant chain and also generate antigenic peptides for HLA class II-mediated antigen presentation in different cells types in humans and mice (Beers et al., 2005; Hsing and Rudensky, 2005; Shi et al., 2000; Tang et al., 2006; Villadangos and Ploegh, 2000). Cathepsin G found in endocytic compartments of a variety of antigen presenting cells (but not monocyte-derived DC) has been implicated in processing of several foreign and self-antigens (Burster et al., 2010).

Insights into antigen cross-presentation provided interesting conclusions that might be of great interest to those studying intracellular pathogens. Savina and colleagues (Savina et al., 2006) showed that antigen cross-presentation by dendritic cells is promoted by attenuation of the activity of proteases found in the phagosome, and by recruitment of NADPH oxidase, a known microbicidal enzyme of neutrophils. They revealed that dendritic cells maintained a neutral and even alkaline phagosomal environment, thereby contrasting to the acidic phagosome usually known of macrophages. This elevated pH occurs through recruitment of NADPH oxidase and production of reactive oxygen species (ROS) in the phagosomal lumen that neutralize the acidic environment. Production of ROS was found to be maintained for several hours, contrasting with macrophages where only short bursts were detected. These studies are revealing that attenuation of proteolysis might diminish the destruction of antigens needed for presentation, thus explaining why dendritic cells are more effective in antigen presentation to lymphocytes than macrophages.

One must not forget the evidence in support of the need for an acidic environment for the activity of several cathepsins. Cathepsin S is, until now the only vacuolar enzyme demonstrated to be necessary for cross-presentation (Shen et al., 2004). This enzyme is one of the few vacuolar cathepsins that remains stable and active in a neutral pH environment (Lennon-Duménil et al., 2002), which fits the above-proposed model for presentation of antigens originating in a neutral pH. On the one hand, this model fits cross presentation through MHC class I pathway, but on the other hand, it does not explain fully the activity of proteolytic enzymes of the MHC class II pathway that function in an acidic medium. A proposed explanation is the diversity of ranges of pH observed inside phagosomes (Butor et al., 1995).

In the same cell, one can observe different kinetics of PI3P accumulation (Henry et al., 2004). Since PI3P is required for recruitment of NADPH-oxidase subunits p40phox and p47phox, this suggests different activities for NADPH oxidase in each phagosome, thus rendering the possibility that different presentation pathways may occur. As previously mentioned, MTB is known to inhibit the activity of PI3P through the bacterial lipid ManLAM and the PI3P phosphatase SapM (see “MTB lipids and proteins in phagosomal maturation arrest”). This may prove of some interest for MTB antigen presentation by dendritic cells.

The modulation of phagosomal maturation by MTB clearly affects the delivery of lysosomal enzymes needed for antigen presentation. Moreover, if we take into consideration the fact that some cathepsins might be delivered to early endosomes, an acidic pH would be necessary to activate those proteases. Even so, CD4 T cells respond to MTB antigens on an antigen specific fashion (Beatty et al., 2001; Ramachandra et al., 2001). We may hypothesized that to some extent, only a minor fraction of MTB-containing phagosomes need to be able to mature and acquire lysosomal proteases for an effective antigen presentation to occur.

Several studies searched for possible relations between cathepsin activity and mycobacteria survival. Studies on THP-1 monocytic cell line revealed that IFN- γ -induced Cat S expression was down-regulated when those cells were infected

with BCG (Sendide et al., 2005). Due to that, MHC class II molecules without peptide antigen load accumulated in the interior as well as on the surface of the cells. Moreover, this phenotype was found to be linked to BCG-induced secretion of IL-10. This reveals an ability of BCG to inhibit peptide loading into class II molecules. In another study, BCG was engineered to express Cat S. This resulted in the recovery of normal surface expression of class II MHC molecules carrying BCG antigens (Soualhine et al., 2007). Remarkably, these bacteria were much more readily killed by THP-1 monocyte-derived macrophages than the wild type bacteria, and the BCG-containing vesicles co-localized significantly with late endosomal markers. Another cathepsin, Cat G was shown to possess bactericidal properties. In THP-1 cells, Cat G was shown to be down-regulated upon MTB infection (Rivera-Marrero et al., 2004), a possible strategy to render MTB bacilli more viable. Still, another research group demonstrated in murine alveolar macrophages that down-regulation of Cat G only occurred at 6 and 12 hours post infection (Srivastava et al., 2007). After 3 and 7 days the opposite effect was observed, with high up-regulation of Cat G expression.

Clear evidence for the requirement of acidification for cathepsin activation was depicted in a study concerning Cat D (Singh et al., 2006). The authors correlated the presence of active form (30-kDa) of Cat D with increased acquisition of the proton pump vATPase by the phagosome in macrophages infected with the attenuated strain H37Ra. The consequence of vATPase accumulation was as expected, an acidification of the phagosomal lumen. This study concluded that during infection with the pathogenic strain H37Rv or BCG, vATPase exclusion and more neutral pH phagosomal environment led to an increase in the levels of the Cat D inactive form (46-kDa). Increased phagosomal acidification and Cat D activity upon infection with H37Ra strain resulted in increased MHC class II antigen presentation-dependent induction of IL-2 in T cells.

Cathepsins have also been shown to play a role in the initiation of innate responses to microbes. Cathepsin K inhibition results in attenuation of inflammatory autoimmune diseases, while its pharmacological or genetic inhibition leads to defective TLR9 (endosomal-membrane TLR) signaling in

DCs, without affecting antigen presentation (Asagiri et al., 2008). Similarly, cathepsins B, F, L, and S were shown to be required for TLR9 responses (Matsumoto et al., 2008) by inducing the proteolytic cleavage required for TLR signaling (Ewald et al., 2008; Park et al., 2008). Unprocessed TLR9 retain the capacity to bind their ligands, yet only the processed forms recruit the signaling adaptor protein MyD88. Concerning cytokine signaling, cathepsins were shown to activate or inhibit certain cytokines. In macrophages, a study found that cathepsin B was required for optimal post-translational processing and production of TNF- α in response to LPS (Ha et al., 2008). This suggests a role for intracellular cathepsin B in vesicle trafficking of TNF- α to the plasma membrane.

Table 1.1. List of cathepsins function in immunity and their inhibitors.

Name	Gene	Catalytic type	Distribution	Function	Inhibitor Cystatin
cathepsin A	CTSA	Serine	Ubiquitous	Protective protein	
cathepsin B	CTSB	Cysteine	Ubiquitous	Early neutrophil, T and B cell apoptosis TLRs signaling and TNF-alpha production Processing peptide-HLA II complexes	Cystatins A, B, C, E/M and SN
cathepsin C	CTSC	Cysteine	Ubiquitous	Activation of granzymes A and B	Cystatins A, B, C, SN
cathepsin D	CTSD	Aspartic	Ubiquitous	Early neutrophil, T and B cell apoptosis Processing peptide-HLA II complexes ECM degradation	
cathepsin E	CTSE	Aspartic	Immune cells	Processing peptide-HLA II complexes	
cathepsin F	CTSF	Cysteine	Ubiquitous	TLRs signaling Processing peptide-HLA II complexes	
cathepsin G	CTSG	Serine	Neutrophils, B cells	IL-8, IL-1beta and TNF-alpha activation IL-6 deactivation Destruction of the autoantigen MBP (in B cells)	
cathepsin H	CTSH	Cysteine	Ubiquitous	ECM degradation	Cystatins A, B, C, D
cathepsin K	CTSK	Cysteine	DCs, epithelial cells	TLRs signaling ECM degradation and bone remodeling	
cathepsin L	CTSL1	Cysteine	Ubiquitous	TLRs signaling Processing peptide-HLA II complexes NK T and CD4 T cells production, neuronal cell death and osteoclastic bone degradation	Cystatins A, B, C, D, E/M, F
cathepsin O	CTSO	Cysteine	Ubiquitous		
cathepsin S	CTSS	Cysteine	APCs	TLRs signalling Processing peptide-HLA II complexes NK T cells production, ECM degradation	Cystatins A, B, C, D
cathepsin V	CTSL2	Cysteine	Ubiquitous	Processing peptide-HLA II complexes NK T and CD4 T cells production	Cystatins E/M
cathepsin W	CTSW	Cysteine	NK and T cells	Processing peptide-HLA II complexes	
cathepsin X	CTSZ	Cysteine	Ubiquitous	T cell activation	

(Rawlings et al., 2014).

1.9. MicroRNA regulation in MTB-infected host cells

Genomic studies have revealed numerous portions of the genome that do not encode proteins, while encoding bioactive non-coding RNA species. This explained why only less than 2% of the genome contains protein-coding genes although more advanced techniques determined that at least 90% of the genome was transcribed (Costa, 2010; Ponting and Grant Belgard, 2010; Stein, 2004). A part of this huge, non-protein coding portion of the genome was shown to encode RNA molecules that regulate the expression of other genes, similarly to another already known process called RNA interference. These small non-coding regulator RNAs were called microRNAs (miRNAs), possessing 19-22 nucleotides long and being involved in post-transcriptional gene expression control (Bartel, 2004) and influencing many biological systems, including mammalian immune systems (Williams, 2008).

Human miRNAs have been shown to be involved in cell development, differentiation, proliferation, apoptosis, DNA methylation, DNA repair and provide anti-inflammatory or pro-inflammatory stimuli (Belver et al., 2010; Small and Olson, 2011). miRNAs are matured and processed in a multistep process involving several enzymes. The primary transcript is transcribed by RNA polymerase II as a several hundred nucleotides long double-stranded transcript called pri-miRNA (O'Connell et al., 2010a). Pri-miRNA folds into a hairpin structure which is poly-adenylated and capped. The RNase III-type enzyme Drosha converts this precursor into a double-stranded miRNA precursor of 60 to 100 nucleotides hairpin known as pre-miRNA (Denli et al., 2004). Pre-miRNAs contain a local stem-loop structure that encodes miRNA sequences which are exported from the nucleus to cytoplasm by exportin 5 (Yi et al., 2003). Pre-miRNAs are then processed in the cytoplasm by the RNase II Dicer in order to yield imperfect, approximately 22-nucleotides long double-stranded miRNA. This duplex is unwound by helicases into two single strands consisting of a guide strand, that will become the mature miRNA, and a passenger strand which will be degraded (Chendrimada et al., 2005). The mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which recognizes specific targets and induces posttranscriptional gene silencing thus regulating protein expression (Khvorova et al., 2003).

Another alternative biogenesis pathway for miRNAs was recently discovered in which following Drosha processing, the pre-miRNA directly enters RISC and skips further processing by Dicer (Cheloufi et al., 2010). The silencing occurs when miRNA-RISC complex binds the miRNA in the complementary region inside the 3' untranslated region (3' UTR) of the mRNA in order to terminate the translation or to lead to degradation of the mRNA (Lu et al., 2005; O'Donnell et al., 2005). Once the miRNA is bound to a completely complementary region the mRNA is degraded. However, miRNA-mediated regulation does not require a perfect match. Only the 7-base sequence between 2nd and 8th nucleotide is called the "seed region," and a complete match of this sequence is required. It is thought that the strength of the inhibition varies depending on the sequence but the precise relevant factors are still to be determined (Singh et al., 2013a). A single miRNA may target hundreds of mRNAs and several miRNAs can also target the same mRNA and result in enhanced translational inhibition (Selbach et al., 2008).

A plethora of miRNAs have been found to regulate key cells of the immune system. Evidences found regulation in T cell differentiation and function (Du et al., 2009; Ebert et al., 2009; O'Connell et al., 2010a, 2010b; Stittrich et al., 2010) and in the innate function of macrophages, DCs and NK cells (Bezman et al., 2010; O'Connell et al., 2010a; Taganov et al., 2006). Moreover, miRNAs have not only been found in tissues but also in serum and other body fluids protected from RNase activity through a stable association with RISC and circulating freely or bound to exosomes.

Pathogenic mycobacteria use the host miRNA to manipulate its response to infection (Table 1.2). A recent study with *M. avium* in human macrophages (Sharbati et al., 2011) revealed several miRNAs to be up-regulated or down-regulated upon infection. Notably, let-8e and miR29a were found to respectively target caspases 3 and 7, major effectors of apoptosis triggering (Fink, 2005), providing more evidence for MTB-induced inhibition of apoptosis. In contrast, in murine T cells infected with *Listeria monocytogenes* or BCG, miR-29 was found to be down-regulated while its target was IFN- γ mRNA (Ma et al., 2011).

Two miRNAs, miR-125b and miR-155, were found to be differentially regulated upon infection of macrophages with MTB or *M. smegmatis*. While miR-125b targets TNF- α mRNA, miR-155 is thought to indirectly enhance TNF- α production by increasing TNF- α mRNA half-life and translation (Tili et al., 2007). During infection with live MTB or simply by treatment with MTB lipomannan there was an induction of miR-125b and reduction of miR-155 expression leading to low TNF- α production. In contrast, when macrophages were infected with live *M. smegmatis* or treated with its lipomannan, miR-155 expression was induced while miR-125b was reduced, leading to high TNF- α production (Rajaram et al., 2011). In DCs, miR-99b was also found to mediate TNF- α generation while its knockdown led to enhanced production of TNF- α with impact in MTB intracellular growth (Singh et al., 2013b). Recent work also found that EsxA (ESAT-6) induced the expression of miR-155, which subsequently repressed Bach1 and SHIP1 expression. Bach1 is a transcriptional regulator of heme oxygenase-1, which has been implicated in activation of MTB dormancy regulon (Dos), while SHIP1 inhibits the activation of a serine/threonine kinase AKT that is important for MTB survival. Another role for miR-155 is mediation of macrophage apoptosis through TLR signaling. BCG were found to trigger miR-155 by a TLR2-dependent mechanism (Ghorpade et al., 2012).

I previously mentioned the important role of vitamin D in enhancement of macrophage-mediated killing of MTB (see “Activators of the mycobactericidal mechanisms of macrophages”). Two vitamin D-dependent peptides, CAMP and DEFB4A were found to be regulated by miR-21, possibly by down-regulation of TLR2/1 heterodimer-induced CYP27B1. Triggering of TLR2/1 was found to induce IL-1 β secretion, increase the expression of IL-1 receptor (IL-1R1) and decrease the secretion of IL-1 receptor antagonist (Liu et al., 2009). Inhibition of this pathway by miR-21 thus resulted in decreased IL-1 β expression and activity, and also indirectly by up-regulation of IL-10 (Liu et al., 2012). Knockdown of miR-21 in *M. leprea*-infected monocytes led to restoration of CAMP and DEFB4A expression and TLR2/1-mediated antimicrobial activity.

More recently, one microRNA, miR-142-3p, was found to interfere with macrophage phagocytosis by targeting the actin-binding protein N-WASP (Bettencourt et al., 2013). This microRNA was differentially regulated by MTB

and *M. smegmatis*. Comparatively with *M. smegmatis*, MTB induced an early up-regulation of miR-142-3p at 1 h in mouse macrophages and 4 h in human macrophages while another study revealed that miR-142-3p was down-regulated later during the infection (24 h post-infection) in mouse macrophages (Martinez et al., 2013). In another study regarding miR-142-3p, the authors found that BCG infection induced early down-regulation of miR-142-3p and that this led to an increase in the expression of IRAK-1 (Xu et al., 2013), an inhibitor of TLR signaling (Ghorpade et al., 2013). These results indicate an increased recognition of BCG and generation of pro-inflammatory response, while MTB avoids it by up-regulating miR-142-3p during the early stages.

Since miRNAs circulate in the body they have the potential to become accessible biomarkers of disease and aid in diagnostic procedures. Distinct miRNA levels have been found in PBMCs (Liu et al., 2011; Wu et al., 2012), serum (Fu et al., 2011) and sputum (Yi et al., 2012) of TB patients as compared with healthy control. Of the various miRNAs, miR-29a, miR-155, miR-155*, miR-125b, miR-3179a and miR-147 may be potential biomarkers for the diagnosis of TB. Still, further validation is required to determine if these are indeed reliable targets and also how specific is their expression signature in relation to the disease. In a study comparing TB and sarcoidosis patients, although significant differences were found in miRNA expression between patients and healthy individuals, both TB and sarcoidosis patients revealed highly similar miRNAs profiles (Maertzdorf et al., 2012).

The medical significance of miRNAs goes far beyond their potential for good biomarkers. miRNA regulation has received increasing interest from the pharmacologic perspective. miRNAs induced in disease might be targets for specific inhibitors while those that are being down-regulated during the disease could receive an exogenous boost to improve the response against the disease. Efforts have been made for the development of miRNA-based drugs, especially for cancer (Takeshita et al., 2010) and hepatitis C virus infection (Lanford et al., 2010). The differentially expressed miRNAs found in response to MTB infection might reveal great potential as future therapeutics, although no clinical trials have been made so far (Singh et al., 2013a).

Although outside of the scope of this thesis, it is worth mentioning that bacteria and particularly mycobacteria also possess their own small RNAs (sRNAs), typically with 50–500 nucleotides in length, known to act as global regulators of cellular phenotypes (Gottesman and Storz, 2011). The first experimental confirmation of sRNAs in mycobacteria was published in 2009, revealing five trans-encoded and four cis-encoded sRNAs in *M. tuberculosis* H37Rv (Arnvig and Young, 2009). There is still a limited number of functionally characterized sRNAs in mycobacteria but they might prove to be interesting medical targets to exploit bacterial mechanisms involved in survival (for a current review of sRNAs in mycobacteria see Haning et al., 2014).

Table 1.2. MicroRNAs in mycobacterial infection

MicroRNA	Species	Target	Cell/tissue	Function
Let-7e	<i>M. avium</i>	CASP3	Macrophages	Anti-apoptotic
miR-125b	<i>MTB</i>	TNF- α	Macrophages	Regulates Th1 response
miR-142-3p	<i>MTB, BCG</i>	N-WASP IRAK-1	Macrophages	Disrupts phagocytosis and TLR signaling
miR-144*	<i>MTB</i>	TNF- α INF- γ	PBMCs T cells	Cytokine signaling and cell proliferation
miR-146a	BCG	IRAK-1 TRAF6	DCs	Suppress Th1 and Th17 response
miR-155	MTB	SHIP1	Macrophages	Enhance TNF- α
miR-181a	<i>M. leprae</i>	SHP2	Macrophages	Dampens T cell activation
miR-21	<i>M. leprae</i>	CYP27B1 IL1B IL-12p35 PDCD4	Skin lesions Macrophages Monocytes	Inhibition of antimicrobial activity
miR-29	BCG	INF- γ	NK and T cells	Regulates Th1 response
miR-29a	<i>M. avium</i>	CASP7	Macrophages	Anti-apoptotic
miR-99b	MTB	TNF- α	DCs	Regulates Th1 response

(Bettencourt, 2012; Mehta and Liu, 2014).

1.10. MTB membrane transport in drug resistance and intracellular persistence

While MTB has evolved several mechanisms to modulate the host immune response it also possesses several defensive structural features that enable its survival in harsh environments. Mediators of membrane transport are key molecules in MTB survival. As previously mentioned, the MTB-membrane is a phenomenal barrier against diffusion of harmful molecules (see “*Mycobacterium tuberculosis*”) (Figure 1.2). However, the cytosol needs to communicate with the extracellular medium to allow the transport of nutrients and other growth requirements of the bacteria. Evolution towards survival in an intracellular niche likely originated specific and highly regulated transport pathways that provide the minimal open doors for the required molecules while restricting the passage of bactericidal factors or provide the bacteria with the ability to extrude harmful molecules and maintain their internal homeostasis. Some of these strategies are not only useful to protect the bacteria against the host response but also to limit the entry of antibiotics or to excrete them out of the cell.

Concerning the entry of bactericidal molecules into mycobacteria, the influx of toxic compounds is significantly restricted by the complex cell wall and lipid bilayer presenting a significant barrier to the influx of antibiotics (Brennan and Nikaido, 1995). The reduction in membrane permeability leads to a decrease drug influx, thus leading to a decrease in intracellular drug accumulation (Niederweis, 2003; Pasca et al., 2005; Viveiros et al., 2003). Porins represent a fast way across the membrane of mycobacteria for the uptake of nutrients and other molecules (Stephan et al., 2005; Wolschendorf et al., 2007). Porins are multimeric non-specific channels first reported in Gram-negative bacteria but present as well in other bacteria, fungi, plants and mammalian cells.

The mycobacteria water-filled channel proteins function in a similar way to the porins of Gram-negative bacteria. They provide passage for water, ions and other small molecules through passive diffusion. Nonetheless, this mechanism of transportation was shown to be very inefficient in mycobacteria. In comparison to the porin pathway of *Escherichia coli*, the porin pathway of mycobacteria is collectively about 1000-fold less efficient (Jarlier and Nikaido,

1990; Stahl et al., 2001). Although there are still no definite answers explaining the reason for this lack of efficiency, evidences point towards a significant lower expression of porins in mycobacteria (Niederweis, 2003). This is correlated to the resistance of mycobacteria to antibiotics such as penicillins, cephalosporins and tetracyclines (Nikaido, 2001) and also to the characteristic slow growth of pathogenic mycobacteria (Sharbatitehrani, 2004; Stephan et al., 2005).

The first porin identified in mycobacteria, MspA, allows hydrophilic compounds to enter *M. smegmatis* (Niederweis et al., 1999). Studies have shown that deletion of the mspA gene leads to reduced membrane permeability towards glucose and phosphates (Stahl et al., 2001; Wolschendorf et al., 2007). *M. smegmatis* has a faster growth rate than slow growing MTB. When mspA was knocked down, researchers verified that the growth rate of *M. smegmatis* was reduced (Stephan et al., 2005). Not only was there less inflow of nutrients, but also the resistance of these bacteria to antibiotics was increased (Danilchanka et al., 2008a). Moreover, in another study, when slow growing mycobacteria such as MTB and BCG were cloned with mspA, their glucose uptake increased and resulted in a faster growth rate in BCG (Mailaender et al., 2004). Also, susceptibility of both bacteria to β -lactam antibiotics, isoniazid, ethambutol and streptomycin increased. MspA porin has also been implicated in increased susceptibility of *M. smegmatis* to intracellular killing by macrophages. Mutants without the mspA gene were more resistant to nitric oxide *in vitro* and also displayed increased survival in macrophages (Fabrino et al., 2009). Furthermore, these porins also facilitate antibiotic inflow into the bacteria. In another study, Niederweis and colleagues generated several BCG mutants using transposon mutagenesis and tested them for antibiotic resistance (Danilchanka et al., 2008a). Recently, an outer membrane protein, CpnT, was identified in MTB and its channel activity was demonstrated in glycerol uptake (Danilchanka et al., 2014). However there is still no evidence for its role in the transportation of other molecules or as a resistance factor.

Concerning efflux mechanisms, efflux pumps are thought to have evolved due to selective pressure to protect the bacteria against intracellular toxic molecules and maintain cellular homeostasis and physiological balance through active transport of toxic metabolites to the extracellular environment (Louw et al.,

2009). Recent evidence suggests that mycobacteria actively extrude many drugs (Lomovskaya and Watkins, 2001; Telenti, 1997; Victor et al., 2002) via active efflux systems (Marquez, 2005; Pasca et al., 2005; Sander et al., 2000). This broad range of efflux of toxic compounds might be a side effect of the transport of other substrates (Choudhuri et al., 1999, 2002; Putman et al., 2000; Zhang et al., 1999). Still, some efflux pumps were shown to be specific of certain types or classes of antibiotics while others are able to expel a variety of structurally different compounds (Kumar and Schweizer, 2005; Lomovskaya and Watkins, 2001; Marquez, 2005). Multidrug efflux pumps can be regulated by induction of their expression by antibiotics (Grkovic et al., 2001, 2002) or they can also suffer mutations in their regulatory regions leading overexpression (Nikaido, 2001).

Previous studies have explored the contribution of efflux mechanisms to antibiotic resistance in MTB, describing several putative efflux pumps of different classes were shown to be involved in the transport of different compounds (Viveiros et al., 2012). Moreover, while these mechanisms allow the bacteria to survive in sub-inhibitory concentrations of antibiotics, spontaneous mutations emerge, leading to the emergence and selection of drug resistant bacteria (Machado et al., 2012). In addition to this genetic resistance, evidence has emerged for non-genetic or phenotypic resistance phenomenon, also referred to as tolerance. This phenomenon is now considered to be mainly due to the up-regulation of efflux pumps and it was demonstrated that this phenotypic resistance could be lowered when a given antibiotic is administered together with an efflux inhibitor. This highlights the contribution of active efflux to the overall resistance level of a drug resistant strain of MTB (Louw et al., 2011; Machado et al., 2012). Development of mechanisms targeting efflux pumps might be a strategy to decrease the development of drug resistance as well as diminish the length of treatment (Adams et al., 2011; Gupta et al., 2013; Machado et al., 2012; Rodrigues et al., 2012).

Several compounds have been considered as potential efflux inhibitors through their ion channel blocker activity. Some of the described inhibitors are phenotiazines: (1) thioridazine, (2) chlorpromazine and its derivative (3) flupenthixol, a high-potency thioxanthene; (4) haloperidol, a butyrophenone

structurally and clinically related with phenothiazines, and (5) verapamil, a phenylalkylamine (Viveiros et al., 2012). Thioridazine and verapamil have been shown to have active efflux inhibition properties and to inhibit the *in vitro* growth of MTB strains alone or in combination with anti-mycobacterial drugs (Amaral et al., 1996, 2007; Machado et al., 2012; Silva et al., 2001). Verapamil has been shown to exhibit the most potent mycobacterial inhibitory activity to date, being able to enhance isoniazid and rifampicin killing activity in MTB clinical strains (Louw et al., 2011; Machado et al., 2012; Rodrigues et al., 2012). This inhibitor was also shown to potentiate the activity of bedaquiline (Gupta et al., 2014), a diarylquinoline recently approved for the treatment of MDR-TB (WHO, 2013b). It has also been shown that the addition of verapamil accelerates the bactericidal and sterilising activities of TB therapy in mice (Gupta et al., 2013). Besides the direct activity of efflux inhibitors on mycobacteria, reports also show that their activity may enhance the killing activity of macrophages and modulate their response to the bacteria.

Beyond their anti-mycobacterial activity, these compounds also induce immune-modulatory responses in MTB-infected macrophages. It was shown that inhibitors of calcium and potassium can enhance the killing of MTB by macrophages (Martins et al., 2008; Ordway et al., 2003). Also, the inhibition of macrophage calcium channels increases calcium influx, consequently activating immune responses against intracellular MTB (Gupta et al., 2009). The evidences revealed by these studies might constitute new strategies to overcome MTB-induced inhibition of calcium-dependent intracellular response (see “MTB lipids and proteins in phagosomal maturation arrest”).

1.11. Thesis Goals

This thesis focuses on enlightening the mechanisms by which MTB manipulates macrophages and DCs to facilitate bacterial persistence and growth. Conversely, with this information we can consider how to manipulate the infected cells to overcome the disease. Ultimately, our goal is to identify relevant molecules for mycobacterial killing that are being suppressed by the infection and then develop ways to restore or enhance their activity.

Our strategy to address the problem began by focusing on the lysosomal killing mechanisms with particular attention to proteolytic cathepsins. Our main working hypothesis relied on the fact that cathepsins are major enzymes in cell homeostasis and participate in pathogen killing and antigen presentation. We thus proposed that due to their relevance, cathepsins would be excellent targets for MTB-mediated manipulation and relevant for MTB persistence in macrophages and dendritic cells. Indeed, our results show that macrophages and dendritic cells infected with MTB show a general down-regulation of cathepsin expression, in contrast to what occurs with *M. smegmatis* infected cells. Consequently, this enhanced the intracellular survival of MTB and also increased the surface expression of HLA class II molecules.

Following these lines of evidence and considering our previous experience in mycobacterial manipulation of the host cells, we proposed that the mechanism behind cathepsin manipulation could involve the host endogenous regulators, miRNAs. We tested this hypothesis, revealing a yet unknown miRNA in regulation of MTB immunity, miR-106b-5p. We show that this miRNA is overexpressed in MTB-infected macrophages, relative to *M. smegmatis*-infected macrophages and that it also regulates cathepsin S expression. The outcome of increased miR-106-5p expression mimicked the effects observed in cathepsin S-depleted macrophages and DCs.

In the third part of our approach, we decided to test pharmacologic molecules described as ion channel blockers to manipulate the endocytic/phagocytic pathways. As summarized in the introduction, these molecules enhance antibiotic treatment by dampening drug efflux, but there are also reports claiming they can act in the host cells themselves, thereby enhancing their bactericidal activity. We proposed that a significant part of the killing enhancement generated by these compounds occurs due to manipulation of the endocytic/phagocytic environment. Our results show that the ion channel blockers lead to a non-specific increase in the acidification of the endocytic compartments; besides the likely direct effect of low pH, the enhanced MTB killing was also due to increased activity of acidic proteases such as cathepsin B.

In the last set of results, we decided to put the focus on the bacteria themselves and their innate mechanism of resistance. In the introduction I mentioned that in spite of MTB-induced manipulation of the host, these bacteria possess mechanisms that allow their survival even when the conditions are potentially very detrimental, limiting the access of harmful molecules to its cytosol and initiating metabolic programs adapted to starvation. In this last part, we proposed that restricted channel activity in MTB is a resistance factor that limits the potential damages caused by molecules released throughout host endocytic pathways and that exploiting this pathway might be important to improve host-mediated killing. We show that a novel channel protein of MTB, CpnT, is a candidate for drugs and host bactericidal molecules. Our results show that the absence of this protein increases the resistance of the bacteria even when we induce strong oxidative stress by IFN- γ -induced release of nitric oxide. Moreover, we also determined that the presence of this protein leads to enhanced pro-inflammatory response, arguing that it is either a recognized PAMP or a pathway for PAMP secretion.

Overall, we delineated a multidisciplinary strategy to study the interaction between the host cells and MTB. Our results revealed (1) specific cathepsins as important molecules in killing and antigen presentation; (2) how MTB uses the host own regulatory miRNAs to reduce the activity of one specific cathepsin, cathepsin S; (3) a pharmacological method to improve macrophage-mediated MTB killing by inducing acidification of the endocytic compartments and enhancing cathepsin activity; (4) an MTB-channel protein that might represent a pathway for diffusion of host bactericidal molecules and recognition by innate receptors.

1.12. Bibliography

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Chapter 2: *Mycobacterium tuberculosis*
manipulation of host cathepsins

David Pires, Joana Marques, João Palma Pombo, Nuno Carmo, Paulo Bettencourt and Elsa Anes

In this chapter, I contributed to the design and execution of all experiments except those with chemical inhibitors as well to the analysis and discussion of the results

***Mycobacterium tuberculosis* manipulation of host cathepsins**

David Pires, Joana Marques, João Palma Pombo, Nuno Carmo, Paulo Bettencourt and Elsa Anes

2.1. Abstract

Mycobacterium tuberculosis resides within phagosomes in host macrophages and dendritic cells and prevent vesicle fusion with lysosomes. A consequence of this inhibition is reduced acidification and proteolysis resulting from less activity of lysosomal cathepsins. Cathepsins are proteolytic enzymes known to participate in the maintenance of cell homeostasis. They are of particular interest in the control of microorganisms through direct pathogen killing as well as by processing of microbial antigens and the antigen presentation machinery itself. In this work, we have screened cathepsin gene and proteins expression in primary human macrophages and dendritic cells and our results indicate that the infection by *M. tuberculosis* induces a general down-regulation of cathepsins expression in infected cells as well as an inhibition of IFN- γ or TNF- α -mediated induction of cathepsin expression. We further reveal that the decrease in cathepsin B, S and L expression and biochemical activity favors bacterial survival, both in macrophages and dendritic cells. The silencing of cathepsins B, S or L in these cells was associated with an increase in the surface expression of class II antigen presentation complex HLA-DR. Overall, we have shown that cathepsins are important for the control of MTB infection and that the bacteria manipulate their expression to favor their survival.

2.2. Introduction

Tuberculosis remains a worldwide health problem with 8 million new cases diagnosed per year and more than 1 million deaths per year as reported by the World Health Organization (WHO, 2013a). The onset of antibiotic treatment led to a great reduction in the prevalence and incidence of the disease particularly in developed countries. Still, the emergence of multi- (MDR) or extremely-drug resistant (XDR) strains of *M. tuberculosis* (MTB), the etiologic agent of TB, has brought renewed attention to the dangers of TB spread with the reports estimating 450,000 new cases of MDR-TB per year (WHO, 2013a). New strategies that synergistically fight the disease not only through antibiotic treatment but also by enhancing the natural ability of the immune system to tackle the pathogen might be the answer to improve MTB clearance and thereby reduce the probability of generating resistant strains.

One of the first encounters of the immune system with the pathogen begins in the lungs where macrophages and dendritic cells internalize the bacteria (Kang et al., 2011). These cells are usually able to destroy bacteria upon phagocytosis by inducing oxidative stress at an early stage and then acidifying the bacteria-containing phagosome by fusion with late endosomes and lysosomes and ultimately inducing the activity of proteolytic and lipolytic enzymes (Russell et al., 2009). These events will lead to pathogen destruction and processing of its antigens to be presented to lymphocytes through the class II antigen presentation machinery. Pathogenic mycobacteria, however, impair these phenomena by blocking phagosome maturation and consequent fusion with late endosomes and lysosomes, avoiding contact with their degradative enzymes (Deretic et al., 2006; Russell, 2001). Still, there is evidence that mycobacteria antigens are presented to lymphocytes and that a fraction of phagosomes fully mature, that is they fuse with lysosomes (Cooper, 2009; Jordao et al., 2008).

The intracellular environment of DCs was shown to be less permissive for MTB growth than the environment in the macrophage. DC-mediated antigen presentation is fundamental for the initiation of cellular immunity and stimulation of MTB-infected macrophages with pro-inflammatory cytokines, such as interferon- γ , secreted by antigen-specific T cells (Cooper et al., 2011; Flynn et

al., 1993; Philips and Ernst, 2012). Besides macrophage activation by pro-inflammatory cytokines, other cellular processes, such as autophagy and apoptosis (Alonso et al., 2007; Behar et al., 2011; Gutierrez et al., 2004; Winau et al., 2006) may overcome the arrest of phagosome maturation and lead to the digestion of the pathogen, suggesting an important role for lysosomal effectors.

Cathepsins are the most investigated proteases. They operate in several cell functions, such as protein processing, pathogen killing, antigen presentation, apoptosis and tissue remodeling (Conus and Simon, 2010). Some of these cathepsins, such as cathepsins B, D, G and S are already known to interact and kill invading microorganisms (Bewley et al., 2011; Hole et al., 2012; Rivera-Marrero et al., 2004; Soualhine et al., 2007; Steinwede et al., 2012). There is also evidence for the role of cathepsins S, F, L and V in antigen presentation by processing the invariant chain linked to class II HLA molecules in several types of cells (Beers et al., 2005; Hsing and Rudensky, 2005; Shi et al., 2000; Tang et al., 2006; Villadangos and Ploegh, 2000). The type of cell infected and its stimulation also results in different kinetics of cathepsin activity. Resting macrophages are thought to be more prone to initiate a strong proteolytic activity upon microbial invasion, that will lead to the destruction of peptidic antigens. By contrast, dendritic cells rely on control of acidification and cystatin expression to reduce this proteolytic response in order to preserve epitopes and thus elicit more efficient lymphocyte priming (Pierre and Mellman, 1998; Savina and Amigorena, 2007; Savina et al., 2006; Yates et al., 2007). The host transcriptional profile induced by MTB is significantly different when the bacteria are inside DCs as compared with macrophages (Tailleux et al., 2008), indicating that comparative studies of the intracellular events and fate of MTB in macrophages and DCs are likely to yield valuable information.

In this study we decided to screen for how cathepsins and their inhibitors cystatins are being regulated in primary human macrophages and dendritic cells during infection with *M. tuberculosis* comparatively with the non-pathogenic *M. smegmatis*. *M. smegmatis* is a non-pathogenic mycobacteria readily killed in macrophages yet sharing a significant number of orthologous genes with MTB (Altaf et al., 2010), making it suitable for comparing and identifying molecules involved in the pathogenesis of tuberculosis. We further analyzed the relevance

of cathepsins and their inhibitors to mycobacteria survival and processing of the antigen presentation machinery. Our results show that *M. tuberculosis* induces a general down-regulatory profile of cathepsins expression in both cell types. We show that this down-regulation leads to increased survival of the bacteria but also to an increase in expression of HLA-DR class II antigen presentation molecules.

2.3. Materials and Methods

2.3.1. Cell lines and culture conditions

Human monocyte-derived macrophages were obtained from healthy blood donors provided by the national blood institute (Instituto Português do Sangue, Lisbon, Portugal). Differentiation of the monocytes into dendritic cells or macrophages proceeded as previously described (Wang et al., 2010). When required, macrophages and dendritic cells were stimulated with 100 IU/ml IFN- γ or 50 ng/ml of TNF- α , respectively, 24 h prior to the infections.

2.3.2. Bacterial Cultures

The strain *Mycobacterium smegmatis* mc²155, containing a p19 (long lived) EGFP plasmid was kindly provided by Dr. Douglas Young (London School of Hygiene and Tropical Medicine, London, UK), and the green fluorescent protein (GFP)-expressing strain of *M. tuberculosis* (H37Rv-pEGFP) plasmid was a kind gift from G. R. Stewart (University of Surrey, United Kingdom). *M. smegmatis* was grown in medium containing Middlebrook's 7H9 Medium (Difco), Nutrient broth (Difco) supplemented with 0.5 % glucose and 0.05 % Tween 80 at 37 °C on a shaker at 200 r.p.m. (Anes et al., 2003). *M. tuberculosis* H37Rv was grown in Middlebrook's 7H9 medium and supplemented with 10 % OADC Enrichment (Difco) (Anes et al., 2003).

2.3.3. Macrophage and Dendritic Cell Infection

Bacterial cultures on exponential growth phase were centrifuged, washed in phosphate-buffered saline (PBS). Bacteria were then resuspended in the desired culture medium without antibiotics. In order to dismantle bacterial clumps, the bacterial suspension was passed through a 21 G needle followed by 5 min ultrasonic bath. Residual clumps were removed by 1 minute centrifugation at 500 x g. Single-cell suspension was verified by fluorescence microscopy. Macrophages and dendritic cells were infected with an MOI of 1 for 3 h at 37 °C with 5 % CO₂. Following internalization, cells were washed three times with PBS and re-suspended in appropriate culture medium without antibiotics.

2.3.1. qPCR

Macrophages and dendritic cells were seeded in 6-well plates at a density of 2×10^6 cells per well. RNA was isolated and purified from infected cells using Trizol reagent (Life Technologies) and following the manufacturer protocol. 1 µg of total RNA was used for cDNA synthesis (Superscript™ II reverse transcriptase, Invitrogen) according to the manufacturer protocol. qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and different sets of primers (Table 2.1) (Eurofins Genomics) at a final concentration of 0.5 µM. The PCR reaction proceeded as follows: 1 cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 sec and, 72 °C for 30 sec. The mRNA expression profiles were normalized with respect to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). The qPCR was performed using an ABI 7300 Real Time PCR System (Applied Biosystems) and data was collected with SDS v1.2 software. The mean of two non-stimulated and uninfected donor samples was taken and used to produce log₂ ratios with respect to the normalized data from stimulated and/or infected cells.

Table 2.1. List of qPCR primers

Target Gene		Target sequence (5'-3')
Cathepsin B	Forward	CCAGGGAGCAAGACAGAGAC
	Reverse	GAGACTGGCGTTCTCCAAAG
Cathepsin C	Forward	CCCCTACACAGGCACTGATT
	Reverse	CAGCTCAAAGGGGTTGAAAG
Cathepsin D	Forward	GACACAGGCACTTCCCTCAT
	Reverse	CTCTGGGGACAGCTTGTAGC
Cathepsin E	Forward	ACTTGACATCCACCCTCCAG
	Reverse	GAATGCCCCAGCCTAACATA
Cathepsin F	Forward	GCCTGTCCGTCTTTGTCAAT
	Reverse	TGGCTTGCTTCATCTTGTTG
Cathepsin G	Forward	GCCTTTCAGGAAAGATGCAG
	Reverse	CACAAAGTCTTCTCGCACCA
Cathepsin H	Forward	ACGGAGGAGTACCACCACAG
	Reverse	GCAATTCTGAGGCTCTGACC
Cathepsin K	Forward	TTCTGCTGCTACCTGTGGTG
	Reverse	GCCTCAAGGTTATGGATGGA
Cathepsin L	Forward	AGGAGAGCAGTGTGGGAGAA
	Reverse	ATCTGGGGGCCTCATAAAAC
Cathepsin O	Forward	AGTGGGACAAACTCCAGCAC
	Reverse	CCTCTTTGATCCCACCTGAA
Cathepsin S	Forward	TCTCTCAGTGCCCGAAGCT
	Reverse	GCCACAGCTTCTTTTCAGGAC
Cathepsin V	Forward	TCCGTGAGCCTCTGTTTCTT
	Reverse	CTAGCCATGAAGCCACCATT
Cathepsin W	Forward	CCACCCCAAGAAGTACCAGA
	Reverse	GTGGCCTTGATCACACCTTT
Cathepsin X	Forward	GGGAGAAGATGATGGCAGAA
	Reverse	ATAGGTGCTGGTCACGATCC
Cystatin A	Forward	GAGGCTTATCTGAGGCCAAA
	Reverse	TCATTTTGTCCGGGAAGACT
Cystatin B	Forward	TGTCATTCAAGAGCCAGGTG
	Reverse	AGCTCATCATGCTTGGCTTT
Cystatin C	Forward	CCAGCAACGACATGTACCAC
	Reverse	ACAGGTGGATTTCGACAAGG
Cystatin D	Forward	GAAGTTCGGTCTGAACCACAT
	Reverse	CCTAGACTTTCCGGCACTTG
GAPDH	Forward	AAGGTGAAGGTCTGGAGTCAA
	Reverse	AATGAAGGGGTCATTGATGG

2.3.2. Western Blotting

Macrophages and dendritic cells were seeded in 6-well plates at a density of 2×10^6 cells per well. Total proteins were recovered with 200 μ l of Laemmli buffer (Sigma-Aldrich). Protein extracts were subjected to electrophoresis in 12 % SDS-PAGE gels, transferred to a nitrocellulose membrane and blocked with 0.1 % Tween 20, 5 % of low fat milk TBS (Tris Buffered Saline). The nitrocellulose membrane was then incubated with primary antibodies specific for human cathepsin S, cathepsins B, cathepsin L and β -tubulin (abcam), overnight at 4 °C. All membranes were washed and incubated with secondary HRP-conjugated antibodies. The bands were visualized with a chemiluminiscence reagent (Merck Millipore) and quantified using ImageJ (Schneider et al., 2012).

2.3.3. Enzymatic activity

Macrophages and dendritic cells were seeded in 6-well plates at a density of 2×10^6 cells per well. Macrophages and dendritic cells were recovered with a 5 mM EDTA/PBS solution. Cell lysis and measurement of the enzymatic activity was performed using an activity fluorometric assay kit specific for each cathepsin (Biovision) or using caspase-1/ICE fluorometric assay kit (Biovision) and following the manufacturer's instructions. The fluorescence intensity was measured with a spectrofluorometer, Tecan M200.

2.3.4. Inhibition of cathepsin activity

Twenty-four hours prior to infection, macrophages and dendritic cells were treated with 20 μ M of inhibitors of cathepsin B, S, L or with cystatin C (Sigma-Aldrich). E-64d, a general irreversible inhibitor of cathepsins (Sigma-Aldrich) was used at 10 μ M after infection due to high cytotoxic effects. Bacteria survival was measured after 24 h of infection.

2.3.5. RNAi silencing

Silencing of cathepsin B, S and L gene expression was performed with Biontex K2® Transfection System. Macrophages were first incubated for 2 h with 4 µl/ml of K2 Multiplier reagent in culture medium. Then, they were incubated with the transfection reagent and 100nM of SMARTpool ON-TARGETplus siRNA (GE Dharmacon) in a ratio of 5 µl_{reagent}:1 µg_{siRNA} for 24 h in antibiotic free medium. Following that, transfection medium was removed and the cells were incubated for 3 days in fresh medium prior to any experiment in order to achieve maximum silencing (data not shown). The transfection efficiency achieved was approximately 95 %, as evaluated by flow cytometry using siGLO Green Transfection Indicator (GE Dharmacon).

2.3.6. Bacteria survival

Macrophages and dendritic cells were seeded in 96-well plates at a density of 5 x 10⁴ cells per well. When required, infected cells were lysed in 0.05 % Igepal solution. Serial dilutions of the resulting bacterial suspension were plated in Middlebrook 7H10 with 10 % OADC (Difco) and incubated for 2-3 weeks at 37 °C before colonies were observable.

2.3.7. Flow Cytometry

Macrophages and dendritic cells were seeded in 24-well plates at a density of 3 x 10⁵ cells per well. Macrophages and dendritic cells were recovered with a 5 mM EDTA/PBS solution and fixed with 4 % paraformaldehyde for 30 min. Following that, cells were stained for 30 min with antibodies specific for human HLA-DR (clone L243, Biolegend) and then analysed in Guava easyCyte™ 5HT flow cytometer.

2.4. Results

2.4.1. Cathepsin profile is cell type- and stimulation-specific

In order to determine the role of cathepsins and their regulators during mycobacterial infection, we started by performing qRT-PCR transcriptomic analysis of cathepsins and cystatins expressed in macrophages and dendritic cells at early stages of infection. We hypothesized that if the infection translates into altered cathepsin expression, those alterations would be measurable as early as 1 day of infection since otherwise their impact in the infection would be less significant. In fact, in another gene expression screen, Tailleux and colleagues (2008) already pointed that while overall gene expression levels change throughout the course of infection the majority of host genes showing regulation induced by MTB in the first 24h remain the same at later time points of infection.

In our analysis we focused on macrophages and DCs infected with *M. tuberculosis* and compared them to macrophages infected with the non-pathogenic *M. smegmatis* at a resting state (M0 macrophages or immature DCs) or following activation with IFN- γ (M1) or TNF- α (mature DCs), respectively. We decided to analyze these cells at different states of activation/maturation since during the pathogenesis of tuberculosis the bacteria will encounter different populations of cells or will potentially be exposed to the effects induced by the cytokines secreted by bystander cells. We began by identifying differences in cathepsin expression between uninfected macrophages and dendritic cells (Figure 2.1). The analysis of immature DCs revealed an up-regulation of cathepsins C, E, O, W, X and cystatins B, C, D while cathepsins L, S and particularly cathepsin K were found to be significantly down-regulated relative to the levels seen in macrophages.

In Figure 2.2 the data are presented by comparing the different conditions within macrophage and DCs. We can observe that stimulation with IFN- γ led to a clearly distinguishable cathepsin profile with up-regulation of cathepsins B, D, E, G, H, K, L, S, V, X and cystatins A and D. The strongest changes induced by TNF- α - maturation of DCs occurred in cystatins expression, with up-regulation of cystatins A and D and down-regulation of cystatins C. Cystatin C down-

regulation was already proposed to be a mechanism to explain the increase of HLA-class II antigen presentation in mature DCs (Pierre and Mellman, 1998). Concerning cathepsins, DC maturation also resulted in up-regulation of cathepsins G and K and down-regulation of cathepsins C, L and V.

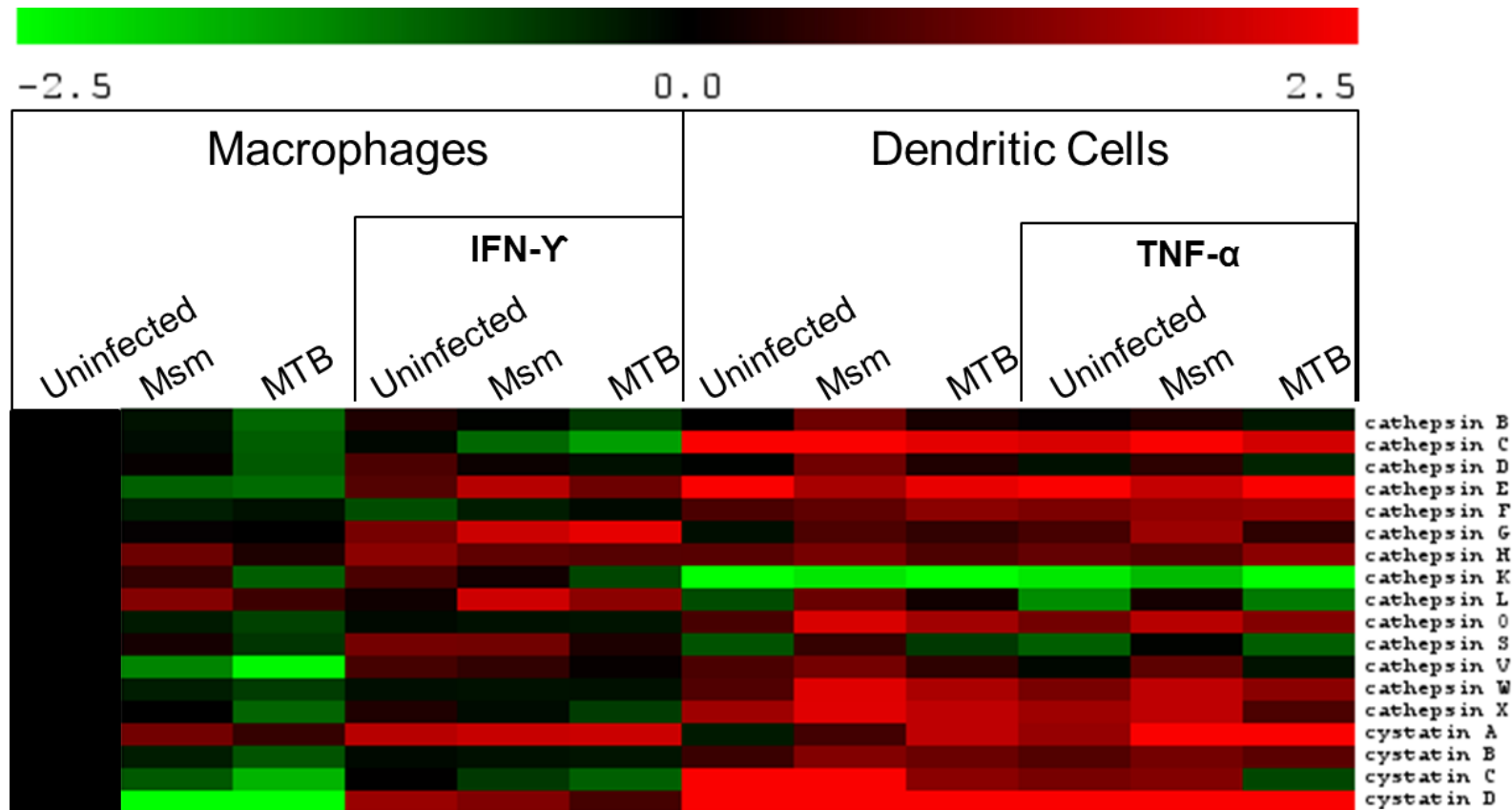


Figure 2.1. Gene expression of cathepsins and cystatins during infection of macrophages and DCs by MTB and *M. smegmatis*.

RNA from macrophages and DCs was recovered after 24 h of infection. When required, 24 h prior to infection, macrophages were stimulated with 100IU/ml of IFN-γ and DCs were stimulated with 50μg/ml of TNF-α. Values are depicted as log₂ gene expression relative to uninfected macrophages. Msm, *M. smegmatis*.

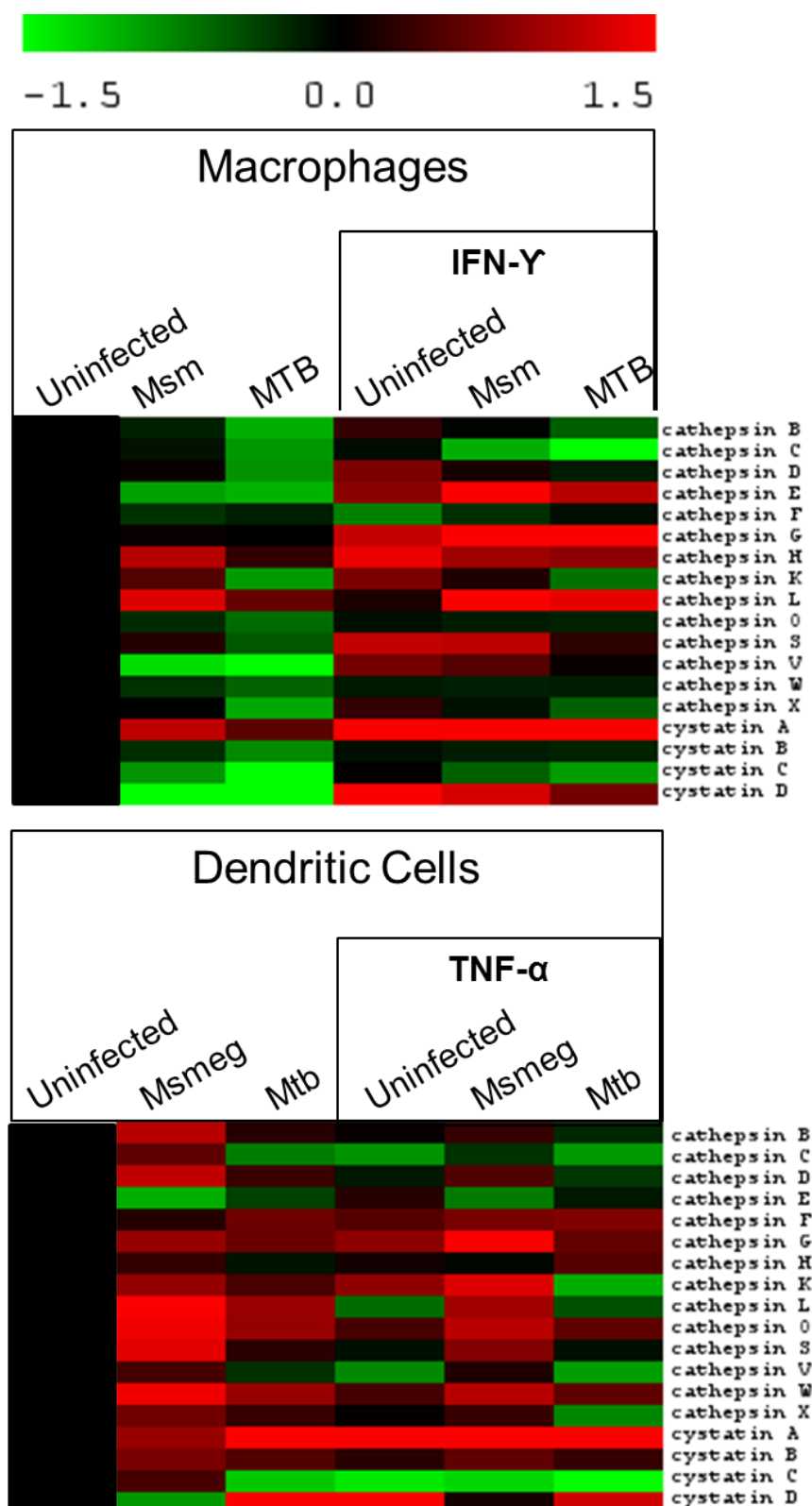


Figure 2.2. Gene expression of cathepsins and cystatins during infection of macrophages or DCs by MTB and *M. smegmatis*.

RNA from macrophages and DCs was recovered after 24 h of infection. When required, 24 h prior to infection, macrophages were stimulated with 100IU/ml of IFN- γ and DCs were stimulated with 50 μ g/ml of TNF- α . Values are depicted as log₂ gene expression relative to uninfected macrophages or uninfected DCs. Msm, *M. smegmatis*.

2.4.2. *M. tuberculosis* induces a specific and cell type-dependent cathepsin expression profile

Next we infected macrophages and DCs with MTB and compared them with *M. smegmatis* infection to highlight the different regulatory phenotypes that they induce and potentially correlate them with MTB pathogenicity or discard them as general phagocytosis phenomena. We determined that infection of macrophages and DCs with MTB or *M. smegmatis* results in different profiles of cathepsins expression, specific of each species (Figure 2.2). MTB infection of macrophages results in the most prominent regulation, leading to a down-regulation of the majority of analyzed cathepsins and cystatins with the exception of cathepsin G, whose expression remained unaltered, and the up-regulated cathepsins H, L and cystatin A. *M. smegmatis* infection also resulted in the down-regulation of several genes though to a lesser extent than MTB when compared to uninfected control cells. The expression levels of cathepsins B, C, D, K, S and X are the most differentially regulated between the two mycobacteria. When macrophages were stimulated with IFN- γ prior to the infection, the expression levels of cathepsins and cystatins were higher than those of non-stimulated cells. The exceptions were cathepsins C, O and W and cystatins B and C, which remained unaltered. Cathepsin F was the only one found to be down-regulated after stimulation. Although IFN- γ increased the expression of most cathepsins, when those cells were infected with MTB, an overall down-regulatory profile was observed. When comparing uninfected and MTB-infected IFN- γ -macrophages, cathepsins B, C, D, K, S, V and cystatin C were the most differentially expressed. Curiously, cathepsin L again showed the opposite effect, being up-regulated upon infection with MTB, similarly to what occurred in non-stimulated macrophages.

In DCs, the infection also resulted in distinguishable patterns of expression (Figure 2.2). *M. smegmatis*-infected DCs showed a general up-regulation of all cathepsins and cystatins with the exception of cathepsin E and cystatins D which were down-regulated. On the contrary, MTB-infected DCs displayed lower levels of cathepsin expression than *M. smegmatis*-infected DCs. Among these, the most differentially expressed between the two bacteria were cathepsins B, C, D, L, S and cystatin C while cathepsins A and D were more

expressed during infection with MTB. When we infected mature DCs (mDCs) with *M. smegmatis* few differences were observed in comparison to infected immature DCs. The exceptions were cathepsin C and cystatin C, which were more expressed in immature DCs, and cystatin D, whose expression was increased in the *M. smegmatis*-infected mDCs. Conversely MTB-infected mDCs revealed several differences in comparison with MTB-infected immature DCs, the majority of which correspond to down-regulation. It is noteworthy that when comparing uninfected mDCs with MTB-infected mDCs, we could only observe differences in the expression of cathepsins K and X. Otherwise the infection appears to be silent concerning cathepsin and cystatin expression.

2.4.3. *M. tuberculosis* inhibits cathepsins B, S and L expression and activity

Cathepsins (Cat) B, S and L are some of the most highly expressed cathepsins in the lysosomes of antigen-presenting cells, and are described as participating in diverse cell functions, such as antigen processing, TLR signaling and cytokine production (Conus and Simon, 2010); these features should render them as obvious candidates to participate in MTB killing and antigen presentation. Our initial screen revealed that these three cathepsins are down-regulated in macrophages and DCs during MTB infection, relative to *M. smegmatis* infection. Accordingly we decided to deepen our analysis of these three cathepsins. In order to understand their relevance in infection we addressed their expression at the level of mRNA, protein and enzyme activity.

We began by analyzing the mRNA expression of these cathepsins at 3, 24 and 48 h post infection (Figure 2.3). In macrophages, we observed that for Cat S and Cat L the differences in gene expression increased throughout the infection while for Cat B, the major difference occurred at 24 h. In DCs we could observe similar results with MTB infection inducing a lower expression of these cathepsins when compared with *M. smegmatis* infected cells at 24 h post-infection. We proceeded by asking whether this down-regulation had any impact on the protein quantity or the activity of these cathepsins (Figure 2.4). Our results show that for all three cathepsins, expression was lowered during

infection with MTB, in comparison with *M. smegmatis*, regardless of the type of cell analyzed. Cathepsin activity also followed this trend (Figure 2.5). For Cat S each species induced an opposite effect, with *M. smegmatis* inducing an increase in activity whereas MTB induced a decrease. For Cat B both species led to reduced activity while MTB had a slightly lower activity than *M. smegmatis*. The activity of Cat L remained constant in macrophages, irrespective of the infection, while in DCs *M. smegmatis* induced a lower activity, and MTB an even more pronounced reduction in Cat L activity.

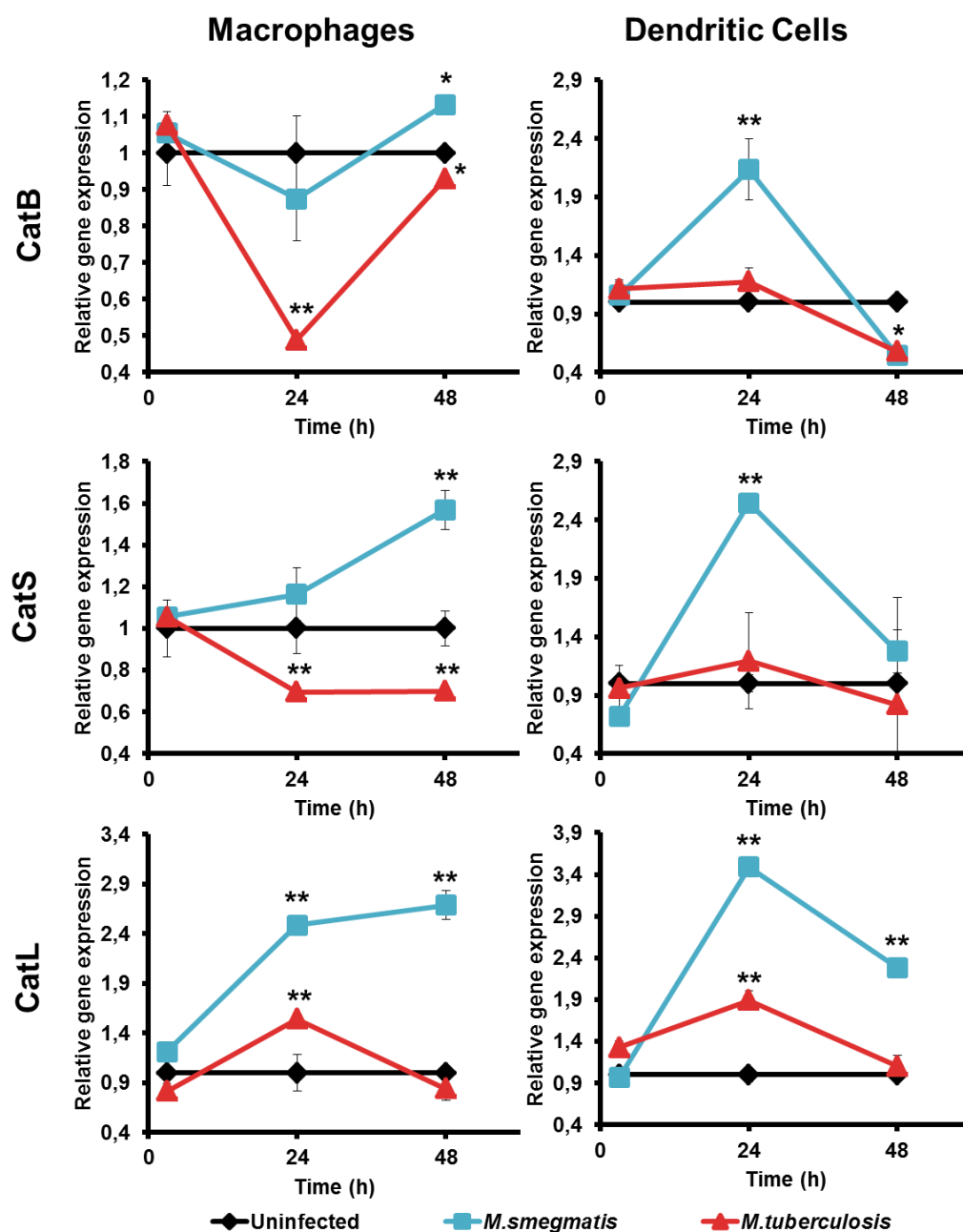


Figure 2.3. Gene expression of cathepsin B, S and L in macrophages and DCs infected with MTB or *M. smegmatis*. Values are depicted relative to uninfected control. (* $p < 0.05$ and ** $p < 0.01$ relative to uninfected control).

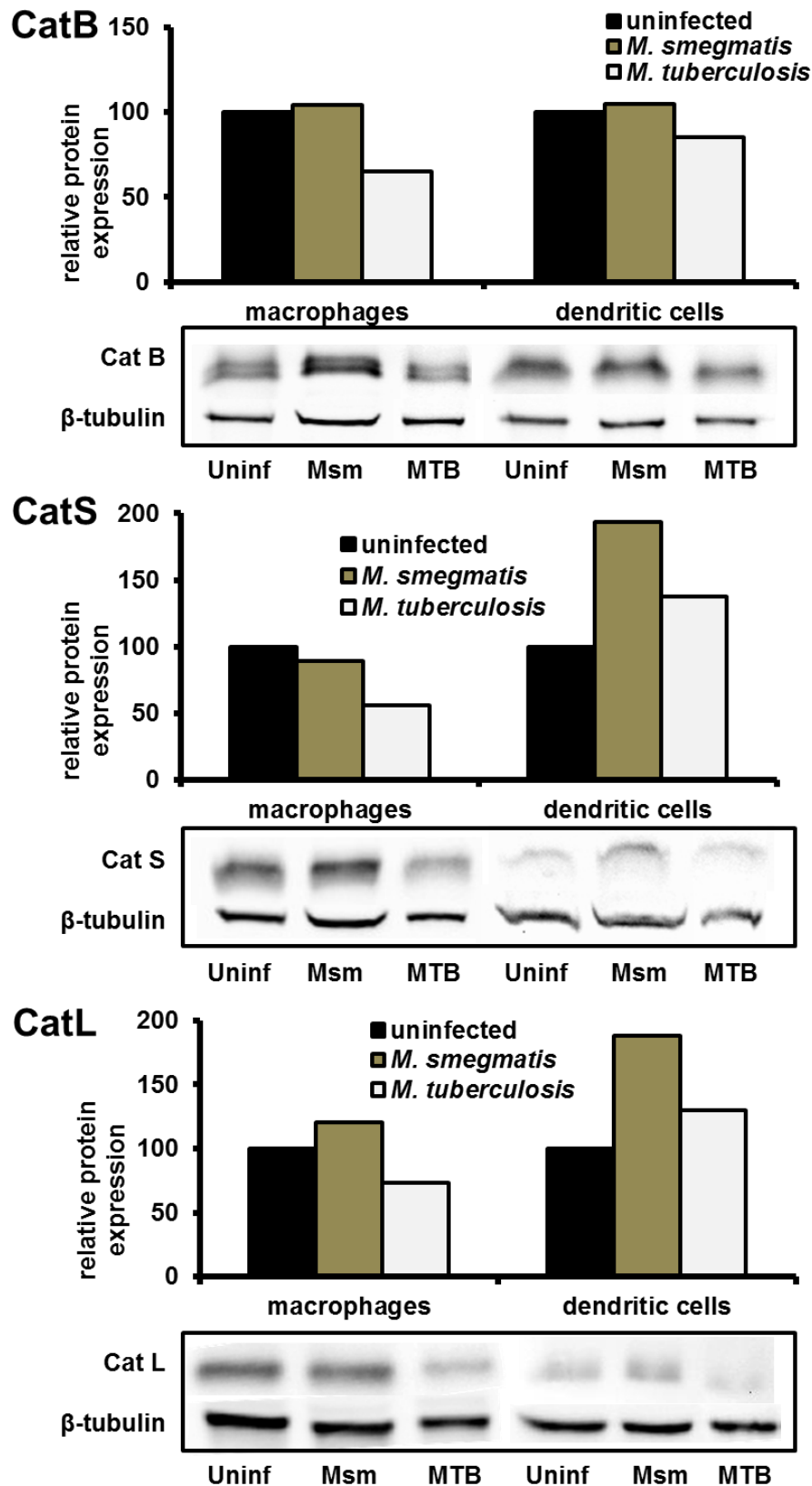


Figure 2.4. Protein expression of cathepsin B, S and L in macrophages and DCs infected with MTB or *M. smegmatis* for 24 h. Bar plots represent protein expression relative to uninfected control and were obtained by densitometry analysis of the western blots.

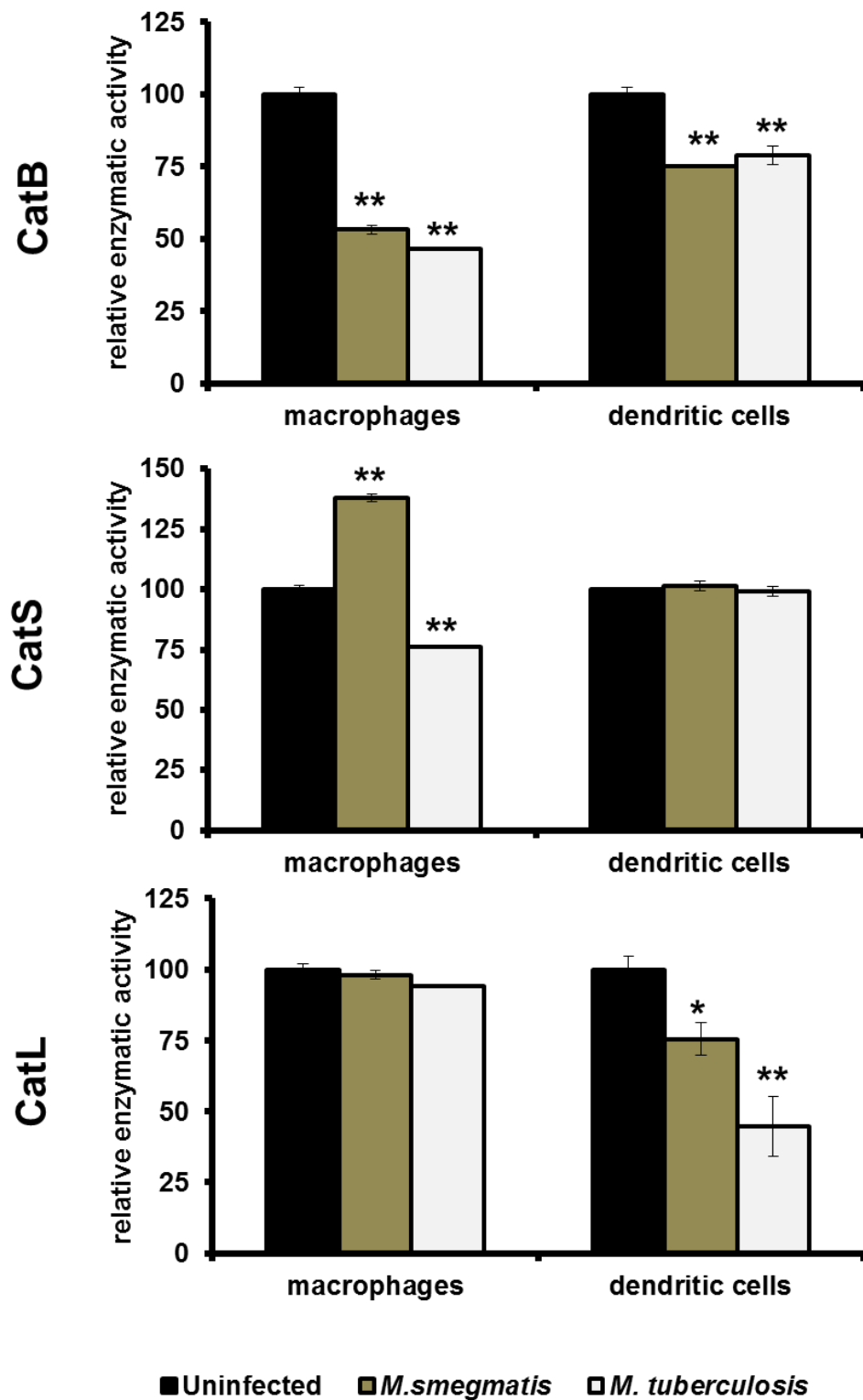


Figure 2.5. Enzymatic activity of cathepsin B, S and L in macrophages and DCs infected with MTB or *M. smegmatis* for 24 h.

Cathepsin activity was measured with a fluorimetric substrate that is activated by cleavage of a cathepsin-specific sequence. Values are depicted relative to uninfected control (* $p < 0.05$ and ** $p < 0.01$ relative to uninfected control).

2.4.4. MTB impairs cathepsin B, S and L up-regulation in activated macrophages and mature DCs.

Macrophage activation with IFN- γ had a different impact on the kinetics of cathepsins expression (Figure 2.6). For Cat B and Cat S, IFN- γ stimulation of macrophages induced an increase in expression at all time points, while *M. smegmatis* expression peaked early during infection, at 3h for Cat B and 24 h for Cat S and then decreased afterwards. For both cathepsins, MTB infection resulted in inhibition of IFN- γ -mediated up-regulation. Concerning Cat L, its expression did not respond to IFN- γ stimulus in uninfected macrophages, yet both bacteria resulted in its up-regulation, with *M. smegmatis* being significantly more expressed than MTB which peaked at 24 h, thereafter showing decrease in Cat L expression.

When TNF- α was used to induce mature dendritic cells, maturation alone resulted in up-regulation of Cat S and Cat L throughout the experiment while Cat B did not display any changes. Infection of these cells with *M. smegmatis* resulted in a peak expression of Cat S and Cat L at 24h with subsequent decrease at 48 h while for Cat B the expression remained the same as that of uninfected cells, dropping afterwards. Once again, MTB infection inhibited these peaks in cathepsin expression and resulted in a down-regulation for the three cathepsins.

Cathepsin protein expression analysis of stimulated macrophages (Figure 2.7) revealed minor changes in uninfected macrophages while cathepsin activity (Figure 2.8) responded to the stimulus with a severe increase of Cat S activity and a slight decrease on Cat B activity. However, infection with *M. smegmatis* or MTB resulted in a drastic reduction of cathepsin expression and activity for the three cathepsins. Regarding mDCs, Cat B showed a minor increase in protein expression only when the cells were infected while that did not translate in an increased enzymatic activity. Cat S expression was up-regulated by 50% due to TNF- α maturation alone yet it became severely up-regulated when mDCs were infected with *M. smegmatis*, an effect that was even more prominent during MTB infection. Still, we could not observe differences in Cat S enzymatic activity. For Cat L, maturation induced a 100% up-regulation of its

protein expression. Furthermore, during infection we could observe a correlation between protein expression and cathepsin activity with a small decrease upon *M. smegmatis* infection and very significant decrease upon MTB infection. It is worth mentioning that for DCs the enzymatic assay might have hindered rapid saturation of the signal, thus explaining the why we could observe down-regulatory effects whereas the up-regulatory effects were unperceivable. This effect might be due to the increased expression of cathepsins in DCs relative to macrophages (Figure 2.1).

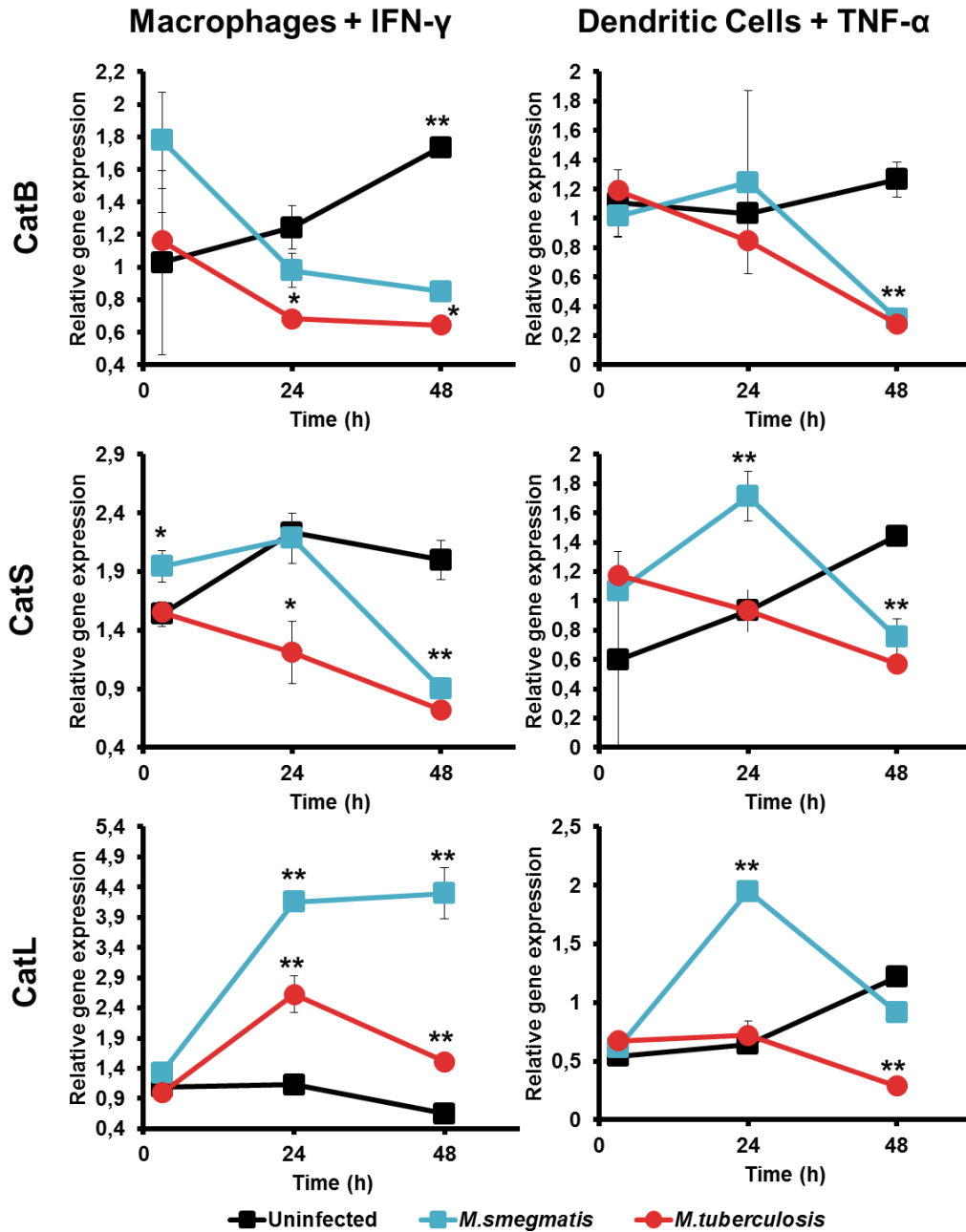


Figure 2.6. Gene expression of cathepsin B, S and L in IFN- γ -treated macrophages and TNF- α -mature DCs infected with MTB or *M. smegmatis*. Values are depicted relative to unstimulated uninfected control. (* $p < 0.05$ and ** $p < 0.01$ relative to uninfected control).

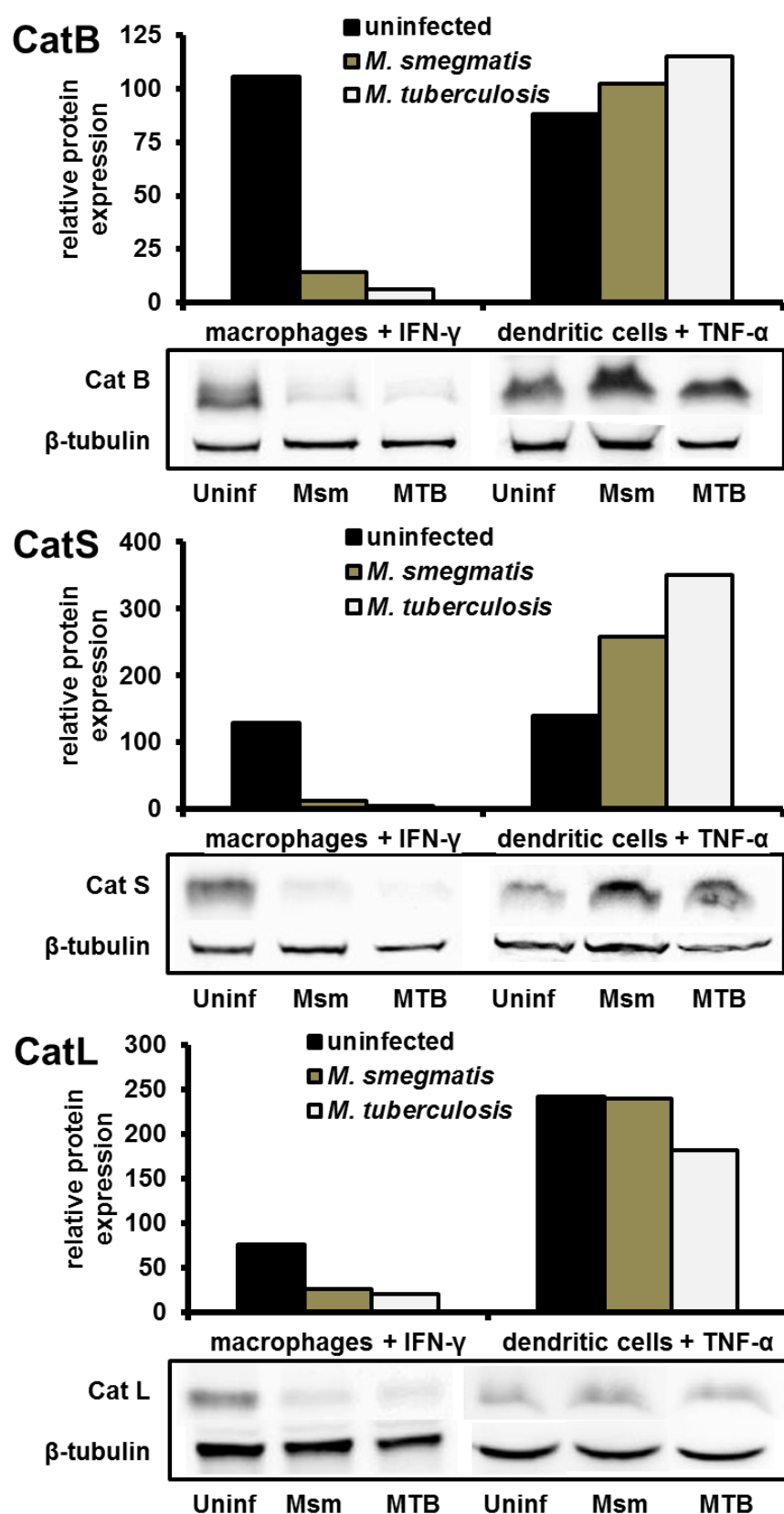


Figure 2.7. Protein expression of cathepsin B, S and L in in IFN-γ-treated macrophages and TNF-α-mature DCs infected with MTB or *M. smegmatis* for 24 h. Bar plots represent protein expression relative to unstimulated uninfected control and were obtain by densitometry analysis of the western blots.

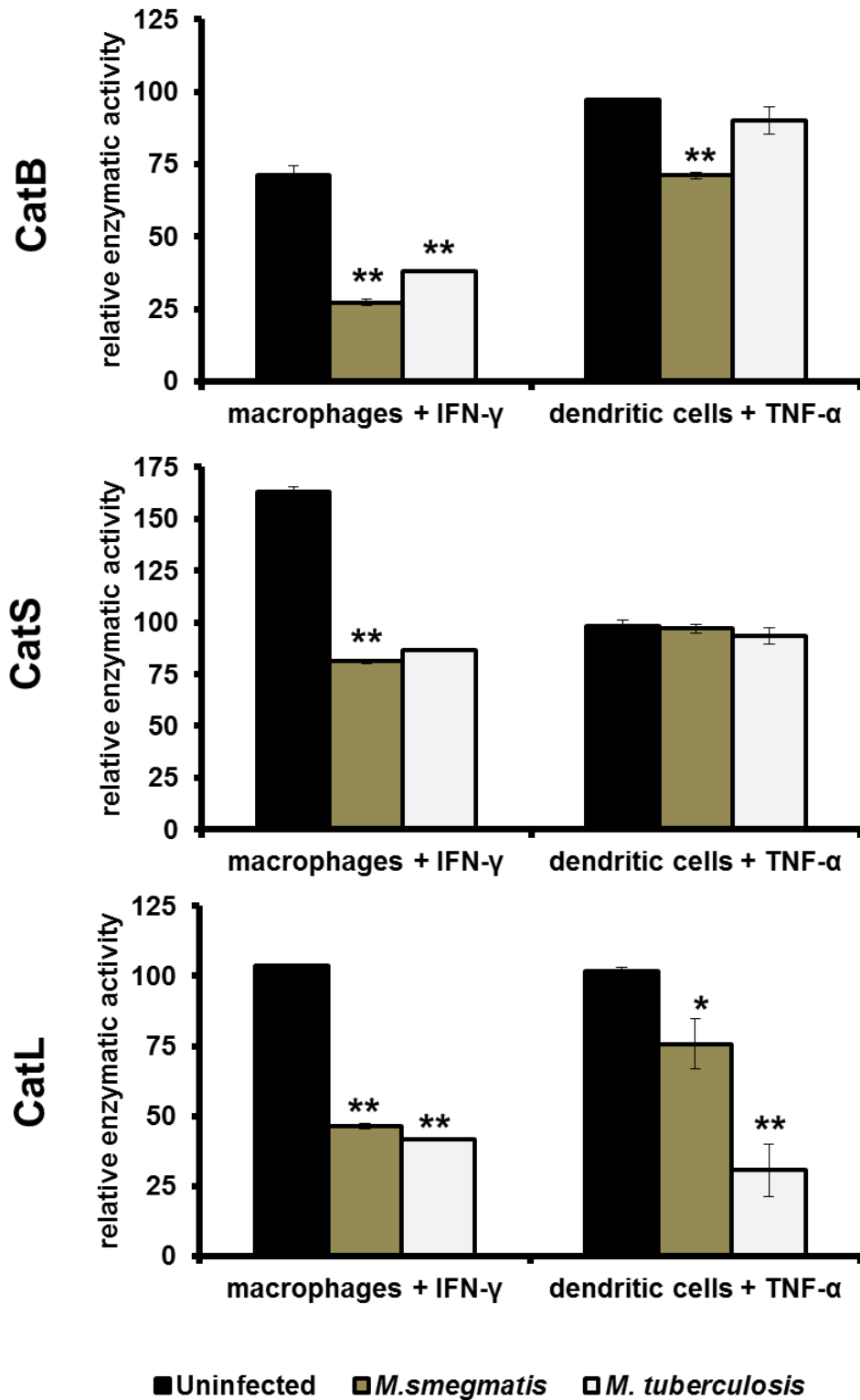


Figure 2.8. Enzymatic activity of cathepsin B, S and L in IFN- γ -treated macrophages and TNF- α -mature DCs infected with MTB or *M. smegmatis* for 24 h. Cathepsin activity was measured with a fluorimetric substrate that is activated by cleavage of a cathepsin-specific sequence. Values are depicted relative to unstimulated uninfected control (* $p < 0.05$ and ** $p < 0.01$ relative to uninfected control).

2.4.5. Inhibition of cathepsin activity with chemical inhibitors or with cystatin C resulted in increased MTB survival in macrophages and DCs

Our previous results revealed that MTB impairs the increase in cathepsin expression and activity that is elicited by non-pathogenic bacteria such as *M. smegmatis*. This prompted us to question if this weakened proteolytic response might benefit MTB survival in its host cells. Our first approach was to use available chemical inhibitors to inactivate cathepsin B, S and L and then measure the consequences on intracellular survival in macrophages and DCs. Moreover, we also tested a general inhibitor of cysteine cathepsins E-64d and cystatin C, the endogenous inhibitor of Cat B, S and L. Prolonged exposure of MTB-infected cells with these inhibitors produced significant cytotoxic effects which compelled us to limit our analysis to the first 24 h of infection. Our results revealed that treatment with chemical inhibitors of cathepsins (Figure 2.9A) results in a significant increase in MTB intracellular survival in IFN- γ -activated macrophages, with a 10-fold increased survival when using Cat B inhibitors and approximately 5-fold increased survival when using Cat S and Cat L inhibitors. In mDCs, Cat L inhibition produced the highest results with a 15-fold increased MTB survival while Cat B or Cat S inhibition resulted in approximately 5-fold increase in intracellular bacilli. The general inhibitor E-64d also produced very significant results in mDCs, yet was not as effective in macrophages as the specific inhibitors. In resting macrophages and immature DCs the inhibitors failed to produce any significant results so we decided to treat these cells with cystatin C (Figure 2.9B). Our results showed that treatment with cystatin C led to a significant increase of MTB survival of 5-fold in macrophages and 3-fold in DCs.

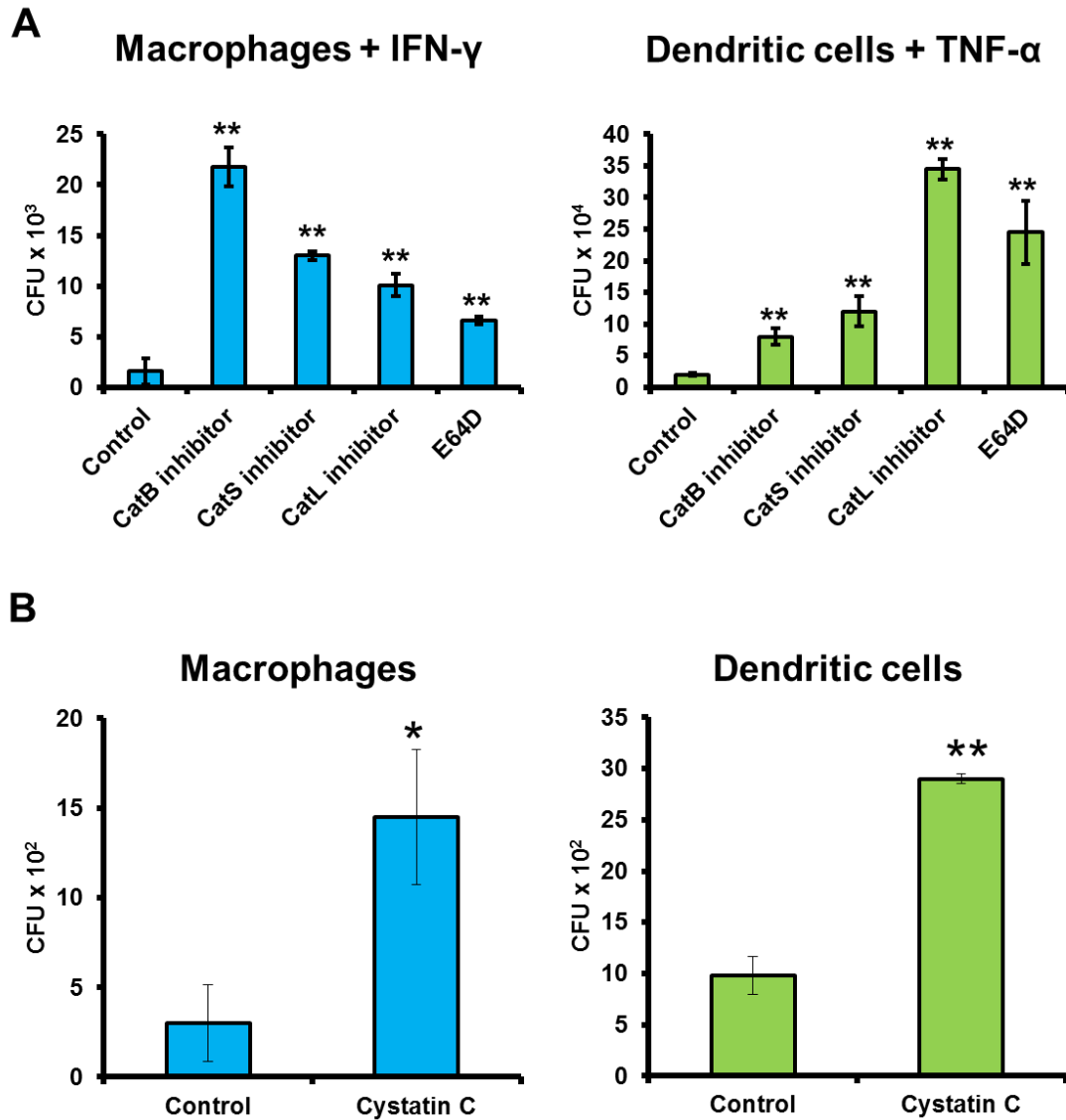


Figure 2.9. Effect of cathepsin B, S and L inhibition on MTB survival. Cathepsin activity was inhibited using 20 μ M of specific inhibitors or cystatin C 24 h prior to infection or 10 μ M of the general cysteine cathepsin inhibitor E-64d after infection. Values are depicted relative to untreated control (* p <0.5; ** p <0.01 relative to untreated control).

2.4.6. Cathepsins silencing results in increased survival of MTB in macrophages

Our results with chemical inhibitors and cystatin C revealed the relevance of cathepsin activity for MTB killing. However, since we were limited to a 24 h analysis due to cytotoxic effects, we decided to employ a more specific inhibition of cathepsin activity by targeting their gene expression by siRNA. We performed this silencing only in macrophages since, for cathepsins the transfection protocol turned out to be very aggressive for DCs, resulting in very few viable cells. Still, we were able to achieve approximately 80% reduction in protein expression and cathepsin activity using this technique in macrophages, which enabled us to analyze the full kinetic of MTB survival throughout 5 days of infection. Our results reveal that all three cathepsins are important for MTB control (Figure 2.10). While the most significant results occurred at day 3 of infection, for Cat S siRNA we could observe a significant increase in MTB survival throughout the entire infection.

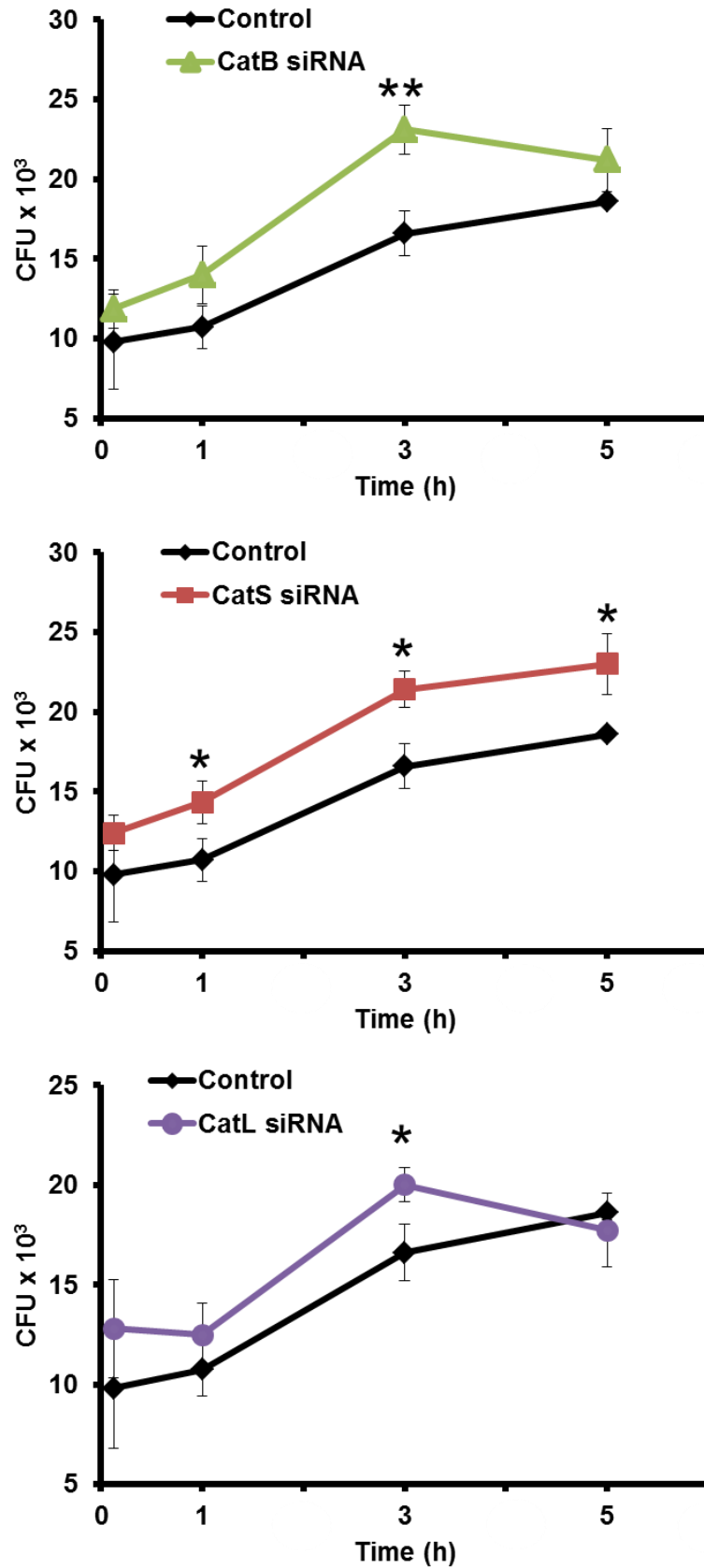


Figure 2.10. Effect of cathepsin B, S and L silencing on MTB survival in macrophages and DCs.

Each cathepsin was silenced by siRNA 3 days prior to infection in order to achieve maximum silencing. (*p<0.05; **p<0.01 relative to control).

2.4.7. Cathepsin silencing increases the surface expression of HLA-DR

Cathepsins are known to participate in protein processing and generation of antigenic peptides to be presented by HLA class II molecules (Rudensky and Beers, 2005). Furthermore, Cat S also participates in the maturation of HLA-class II molecules (Beers et al., 2005). So far, we have established that not only cathepsins are negatively regulated by MTB but they are also important for the control of intracellular infection. Following this evidence we decided to explore the impact of cathepsin silencing in antigen presentation. For this, we applied a similar methodology used for screening molecules involved in HLA-class II antigen presentation, as described elsewhere (Paul et al., 2011). We measured the surface expression of HLA-DR by flow cytometry on cathepsin-silenced and infected macrophages and DCs. First we observed that *M. smegmatis* induced a higher expression than MTB of HLA-DR in both cell types, indicating that MTB inhibits antigen presentation via HLA-DR. Second, by comparing the histograms of macrophages and DCs our results showed that basal levels of HLA-DR surface expression is much higher (≈ 10 -fold) on DCs than on macrophages suggesting that DCs are the major antigen presenting cells responding to MTB infection. Third, silencing of cathepsin expression in both cell types resulted in an increased surface expression of HLA-DR. This was statistically significant for all three cathepsins in macrophages, but only for Cat S in DCs, suggesting a common unspecific mechanism.

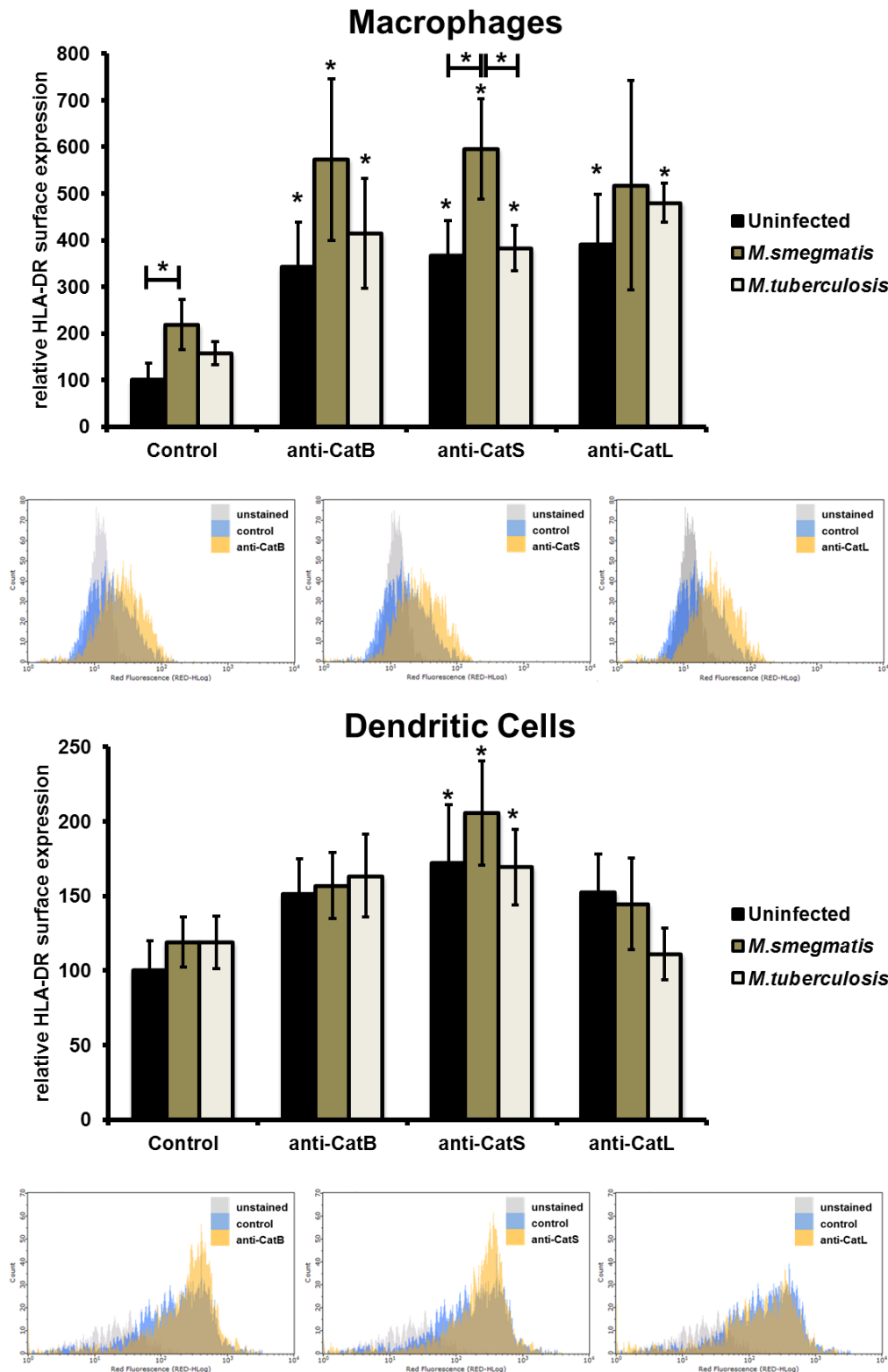


Figure 2.11. Effect of cathepsin B, S and L silencing on HLA-DR surface expression in macrophages and DCs.

Each cathepsin was silenced by siRNA 3 days prior to infection in order to achieve maximum silencing. After 24 h of infection macrophages and DCs were recovered and analyzed by flow cytometry. Values in bar plots depict mean fluorescence intensity of the population (* $p < 0.5$, relative to control or pairwise as indicated above the bars). Fluorescence intensity histograms of MTB-infected cells are presented below the bar plots.

2.5. Discussion

2.5.1. Cathepsins are differentially regulated in macrophages and DCs

Lysosomal proteolysis is a crucial cell process for maintaining homeostasis. Proteolytic enzymes participate in the recycling of cell components, elimination of toxic molecules, processing of other host proteins and digestion of up taken peptides by phagocytosis (Turk et al., 2012). MTB is an intracellular parasite par excellence, and is able to persist by resisting and avoiding the degradative mechanisms of the endocytic pathways. The type of cell which MTB infects produces different responses that are adequate for their general functions, and for stimulation of bystander or primed cells surrounding it. Comparing macrophages and DCs, our results show a lower expression of cathepsins L, S and K. Cathepsins L and S are described to participate in antigen processing or in HLA-class II maturation (Rudensky and Beers, 2005). In dendritic cells, these processes were proposed to be tightly regulated to avoid the destruction of immunogenic peptides and thus improve the efficiency of their presentation to T cells (Savina and Amigorena, 2007).

Another example of DC regulation is shown by the increased levels of cystatin C in immature DCs. This cystatin is a major inhibitor of cathepsin S. It is well established that immature dendritic cells possess high phagocytic activity yet express few antigen presentation molecules at their surface (Driessen et al., 1999). The opposite is true when these cells are matured by stimulation with pathogen-associated molecular patterns (PAMPs) or by TNF- α treatment. As we observed, DC maturation led to a significant decrease in cystatin C expression which is in accordance with the model proposing that this reduction leads to the activation of cathepsin S and processing of HLA class II complexes (Pierre and Mellman, 1998; Zavasnik-Bergant et al., 2005). Moreover, our analysis of the kinetics of cathepsins B, S and L, shows that although maturation resulted in an early decrease of expression it was immediately followed by a time-dependent increase. On one hand, these results show that maturation switches the cell tend towards an increased expression of cathepsins involved in antigen presentation and lower expression of an inhibitory cystatin. On the other hand, this increase in expression need not

translate into more activity, since other forms of regulation, such as pH-dependent regulation will keep their activity under control avoiding over-degradation of antigenic peptides (Savina and Amigorena, 2007; Savina et al., 2006).

The differential expression of cathepsin K was very significant between macrophages and DCs. Cat K is known to be expressed by macrophages, participating in extracellular matrix remodeling and bone resorption. Cat K was also described to be expressed in multi-nucleated macrophages in pulmonary lesions from patients suffering from sarcoidosis, tuberculosis or with granulomas caused by foreign materials (Bühling et al., 2001). This role of macrophages in lung remodeling might serve in the future as a potential biomarker of disease (Reghellin et al., 2010). Macrophages activated with IFN- γ showed a severe increase in the expression of several cathepsins, possibly related with their role in antimicrobial killing. Yet, similarly with DCs, pH-dependent regulation induced by the potent oxidative response of these cells is thought to moderate cathepsin activity (Yates et al., 2007), so this increase in expression might not result in a higher proteolytic activity. Accordingly, our results show that although Cat B gene expression is highly increased upon macrophage activation, the proteolytic activity is decreased by 75 %. Cat S is known to maintain stability and activity even at neutral pH (Brix et al., 2008). Beers and colleagues (2003) have already revealed that IFN- γ stimulation of macrophages results in increased processing of HLA class II-invariant chain complex. Our results are in concordance with this, since we show that Cat S overall proteolytic activity increases by 60% in activated macrophages and thus arguing that this cathepsin is a relevant contributor to proteolysis in a less acidic phagosome.

2.5.2. MTB inhibits pathogen-induced cathepsin expression

The role played by cathepsins in protein processing and digestion suggest their activity might be important for MTB killing and processing of its antigens. MTB improves its intracellular survivability by inhibiting cellular processes that may lead to its destruction, such as phagosome maturation and fusion with

lysosomes. Still, depending on the route of entry, recognition and external stimuli, a portion of the MTB-phagosomes fully mature (Armstrong and Hart, 1975; Brooks et al., 2011; Jordao et al., 2008) leading to the recruitment and activation of lysosomal cathepsins. Moreover, some of these cathepsins are already active in early endosomes (Rudensky and Beers, 2005) suggesting they may come in contact with MTB even when phagosome maturation is arrested.

We propose that one of the mechanisms that MTB uses to persist inside host cells is by avoiding the degradative action of lysosomal cathepsins by inhibiting their expression and/or impairing their activity. Our results show that MTB infection of host macrophages and DCs results in a general down-regulation of cathepsin expression when compared to the regular cathepsin expression profile elicited upon infection with the non-pathogenic *M. smegmatis*. The strongest effect was visible in macrophages where all cathepsins were down-regulated relative to *M. smegmatis* infection. Macrophage response to *M. smegmatis* included an overexpression of cathepsins K, L, S and also cathepsin H. Cat H has been detected in early endosomes and phagosomes of the mouse macrophage cell line J774 (Claus et al., 1998) and was found to become concentrated in late endosomes of mature DCs (Lautwein et al., 2002) suggesting its relevance in antigen processing.

The major down-regulations promoted by MTB compared to *M. smegmatis* in macrophages included cathepsins B, D and S. Cat B and Cat D have been shown to regulate cell death in response to microbial infection by inducing pyroptosis in response to inflammasome activation (Cat B) (Mishra et al., 2010; Willingham et al., 2007) or by inducing apoptosis (Cat D) (Bewley et al., 2011). By inhibiting cell death during the first stages of infection, MTB is able to replicate in its intracellular niche, generating a higher burden of bacilli before erupting from a necrotic cell. In DCs our results produced the clearest distinguishable pattern of cathepsin expression between the two bacteria. *M. smegmatis* was a potent inducer of cathepsin expression while MTB prevented or diminished this increase. Cathepsins B, C, D, L, S and cystatin C were the most differentially expressed between bacteria. A strong decrease in cathepsins B, S and L activity was observed when activated macrophages were infected either with MTB or *M. smegmatis*. Again, this might be a general mechanism to

reduce proteolysis in activated macrophages in order to preserve peptide antigens to be presented. However, Yates and colleagues (2007) described that although in activated macrophages this reduction might be dependent on pH regulation, when activated macrophages were stimulated with LPS the phagosomes readily acidified. This indicates another type of regulation. In fact, our results show that this regulation appears to occur at the protein level, since we observed a strong depletion of cathepsins B, S and L. A reduction in Cat L protein expression has already been described by Nepal and colleagues (2006) in response to infection of IFN- γ -stimulated mouse bone marrow macrophages with live or heat killed MTB and *M. avium*. This is in accordance with our data showing that even the infection with the non-pathogenic *M. smegmatis* could induce this depletion of CatL. Still this group failed to obtain these results for CatB and CatS.

In mature DCs, infection with *M. smegmatis* further stimulated cathepsin expression at 24 h post infection. Yet, the phenotype was very similar to that of infected immature DCs. This might indicate that *M. smegmatis* infection is a good inducer of DC maturation. On the contrary, MTB infection of mDCs resulted in almost no alterations in cathepsin expression when compared to uninfected mDCs. MTB is described to inhibit antigen presentation (Ramachandra et al., 2001) and this silencing phenotype might be another indication of MTB's attempt to avoid triggering adaptive immunity. While measuring cathepsin activity in DCs we encountered some drawbacks related to saturation of signal. Cathepsin activity appeared to be higher in dendritic cells than macrophages, and this led to quick processing of our fluorescent activity probes. While we could still quantify those cases where infection or stimulus induced decreased activity, such as those of Cat B and Cat L, for the situations where stimulation occurred, our measurements likely represent an under-estimation of the true values.

2.5.3. Cathepsins B, S and L are required for the control of MTB infection

Our previous results shown that while *M. smegmatis* stimulates cathepsins expression, MTB induces a lower expression. We wondered if this down-

regulation would have a direct impact in the intracellular survival of MTB. We approached this problem by inhibiting cathepsin activity through a diverse set of techniques, using specific chemical inhibitors of Cat B, Cat S and Cat L, a general inhibitor of cysteine cathepsins E-64D, their natural inhibitor cystatin C, and also by silencing their gene expression with siRNA. All these methods converged to the same conclusion, that inhibition of cathepsins B, S and L results in increased survival of MTB. It is still noteworthy that we could only obtain statistically significant results with the chemical inhibitors in activated macrophages and mDCs. This might be due to the decrease in cathepsin activity observed when infecting those cells. That effect, coupled with chemical inhibition would possibly produce a drastic ablation of cathepsin activity that could be more easily measured. However, in mDCs a decrease in cathepsin activity was less evident. Nonetheless, inhibition with cystatin C proven to be a very effective way to increase MTB survival whereas our use of siRNA silencing allowed us to profile this increased MTB survival throughout 5 days of infection. Altogether, our results consistently show that cathepsins B, S and L are relevant for the intracellular control of MTB infection in macrophages and DCs.

2.5.4. Silencing of cathepsins B, S and L increases the surface expression of the HLA class II complex, HLA-DR

Cathepsins are involved in antigen processing and in the processing of the antigen presentation machinery itself. Cathepsins S and L have been implicated in processing the invariant chain bound to HLA class II complexes. Furthermore, Cat L was also shown to be required for the production of peptide antigens to be presented to CD4 T cells (Honey et al., 2002; Nakagawa, 1998). By analyzing the surface expression of HLA-DR we intended to disclose possible defects in the maturation of HLA class II molecules, as was previously demonstrated in an siRNA screen in a human melanoma cell line (Paul et al., 2011). We observed MTB inhibition of HLA-DR expression comparatively with *M. smegmatis*, an observation already described in several reports (Hmama et al., 1998; Pai et al., 2003; Pennini et al., 2006; Sendide et al., 2005; Wang et al., 2005). Still, we could only observe this in macrophages, where *M.*

smegmatis induced a significant increase in HLA-DR while MTB did not. Previous studies related TLR signaling with HLA class II expression (Harding and Boom, 2010; Pai et al., 2003, 2004; Pennini et al., 2006; Wang et al., 2005). In macrophages, chronic stimulation (15-24 h) of TLRs induced a down-regulation of class II transactivator (CIITA), the main transcriptional regulator of HLA class II molecules, resulting in their decreased expression. In DCs however, TLR stimulation induces maturation and increased HLA class II expression due to post-translational increased stability of class II molecules (Cella et al., 1997b; De Smedt et al., 1996). The differences between these two cell types might account for why we could only observe a significant impairment of HLA-DR induction comparatively with *M. smegmatis* in macrophages.

However, contrary to what we were expecting, when we silenced cathepsins B, S and L we saw an increase in HLA-DR expression in infected and uninfected cells. Our results show a significant 3-fold increase of HLA-DR expression in macrophages silenced for the three cathepsins while in DCs, only Cat S yielded significant results. In fact, previously reported results showed that BCG-infected macrophages attenuate HLA-DR surface expression by inhibiting Cat S expression via IL-10 expression (Sendide et al., 2005). While we have observed a reduction in Cat S in MTB-infected macrophages concomitantly with an attenuation of HLA-DR expression, our results show that these events are not related and that this inhibition is not Cat S-dependent. Another study engineered a Cat S-expressing BCG (Soualhine et al., 2007). They were able to reverse MHC class II inhibition with this strategy, yet the bacilli showed a 10-fold reduction in survivability and depicted increased phagosome-lysosome fusion. This might explain why these studies observed recovery of HLA-DR expression when adding or expressing Cat S. Our results revealed that Cat S is important for MTB-killing and thus, increasing its expressing might also increase phagosome maturation and the availability bacterial antigens to be presented. Moreover, both studies relied on BCG, and the intracellular fitness of these bacteria is significantly lower than MTB, which might explain our discrepant results.

The reason why cathepsin silencing leads to an increased HLA class II expression is likely to be related to unspecific alterations in the proteolytic

environment of the phagolysosome, since we could observe a similar phenotype for the three cathepsins. One explanation might be that decreased proteolytic activity will lead to an increase in the HLA class II pool by limiting the recycling of these molecules. In fact, as mentioned above, in DCs TLR stimulation results in maturation and increase in HLA class II molecules and increased stability of these molecules was proposed to be crucial for this to occur (Askew et al., 2000; Cella et al., 1997b). On the contrary, macrophages show the opposite regulation and this was proposed to be a defense mechanism against an exacerbated induction of Th1 cellular immunity at the infection locus (Harding and Boom, 2010). By silencing important lysosomal cathepsins we might be interfering with normal molecular turnover, explaining the increase in HLA class II surface expression. Further studies should focus on analyzing what is occurring throughout the HLA class II pathway to identify the steps where this deregulation of cathepsin activity occurs. It is also important to evaluate the impact of cathepsin S on other HLA class II molecules and to determine the consequences in T cell priming; this increase might or might not have functional repercussions in antigen presentation since we do not yet know whether or not the HLA complexes are being properly processed.

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**Chapter 3: *Mycobacterium tuberculosis*
manipulates cathepsin S expression by up-
regulating miR-106b-5p in macrophages**

David Pires, João Palma Pombo, Nuno Carmo, Paulo Bettencourt and Elsa Anes

In this chapter, I contributed to the design and execution of all experiments as well as the analysis and discussion of the results.

***Mycobacterium tuberculosis* manipulates cathepsin S expression by up-regulating miR-106b-5p in macrophages**

David Pires, João Palma Pombo, Nuno Carmo, Paulo Bettencourt and
Elsa Anes

3.1. Abstract

Tuberculosis is a global health problem made worse by the increased incidence of drug resistant strains, prompting an urgent need for the development of new strategies to kill the etiologic agent, *Mycobacterium tuberculosis*. The success of *M. tuberculosis* relies on the ability to manipulate host gene expression pathways related with their intracellular survival. Among these, we recently investigated the manipulation of miRNAs that regulate immunity-related molecules of the host and increase the success of the pathogen. Devising approaches to control miRNAs during the infection with *M. tuberculosis* might favor the host response and improve clearance of the pathogen, limiting the emergence of drug resistant strains. Here, we propose that miR-106b-5p is actively manipulated by *M. tuberculosis*. This miRNA is being up-regulated upon infection and this leads to an increased, the survival of the bacteria. We also show that up-regulation of this miRNA targets cathepsin S expression, an important lysosomal protease involved in bacterial killing and processing of bacterial antigens and the antigen presentation machinery.

3.2. Introduction

Mycobacterium tuberculosis (MTB) is the causative agent of tuberculosis and one of the greatest worldwide killer diseases due to a single infectious agent. Although only a small portion of infected individuals will develop tuberculosis,

the fact that one third of the human population is estimated to be latently infected with the bacteria leads to a staggering 8.6 million people developing tuberculosis in 2012 and a worldwide prevalence of 12 million diseased (WHO, 2013a). In spite of these numbers, the global effort to stop tuberculosis has yielded significant results, with a decrease of 2% in TB incidence in 2012. The greatest threat emerging from this disease is the emergence of drug-resistant strains resulting in multidrug resistant TB (MDR-TB) which accounts for approximately 3.6% of the new TB cases and 20.2% of re-incident cases (WHO, 2013a). This fact prompted us to address the problem of tuberculosis from a different perspective than antibiotic treatment. Since in most cases the bacteria are contained, understanding the immune factors relevant for this control might provide us with mechanisms to improve immune-dependent pathways that will help TB treatment and reduce the emergence of drug-resistant strains.

One of the hallmarks of *M. tuberculosis* pathogenesis is its ability to persist in host immune phagocytes such as macrophages (Russell et al., 1997). This persistence is enabled by the control of host cells functions, particularly those involved in phagosome maturation and fusion with lysosomes. Usually these processes lead to the acidification of the phagosome and activation of lysosomal enzymes such as proteases and lipases (Russell et al., 2009), but during MTB infection these events are halted and the bacteria persist in a less degradative environment (Deretic et al., 2006; Jordao et al., 2008; Russell, 2001).

So far, several mechanisms and molecules have been proposed for this ability of MTB to survive in macrophages. MicroRNAs are predictable intermediates in this process due to their wide regulatory role. These molecules are small, non-coding regulator RNAs with 19-22 nucleotides long and are involved in post-transcriptional gene expression control (Bartel, 2004). They silence their targets expression by forming complexes with the RNA-induced silencing complex (RISC) and then binding their 7 nucleotide-long “seed region” with a complementary region of mRNA, leading to termination of translation and/or mRNA degradation (Lu et al., 2005; O'Donnell et al., 2005). There are already several studies proposing modulation of miRNAs by mycobacteria in order to

increase the success of their infection by impairing the release of pro-inflammatory cytokines (Ma et al., 2011; Rajaram et al., 2011), controlling phagocytosis (Bettencourt et al., 2013), preventing cell recruitment to the locus of infection (Dorhoi et al., 2013) and miRNAs are also proposed as biomarkers of TB disease (Fu et al., 2011; Maertzdorf et al., 2012; Meng et al., 2014; Miotto et al., 2013; Wang et al., 2011; Yi et al., 2012).

In this study, we followed up on two of our previous works that showed that MTB is able to inhibit cathepsin up-regulation upon infecting macrophages (see “Chapter 2”) and identified the impact of mycobacterial infection on miRNA expression in macrophages (Bettencourt et al., 2013). We propose that the regulation of one of those cathepsins, cathepsin S, is under the control of a novel miRNA involved in MTB immunity, miR-106b-5p. We show that the expression of miR-106b-5p is being specifically up-regulated by *M. tuberculosis* infection relative to the non-pathogenic *M. smegmatis*. Our data show that the consequence of this manipulation is an increase of the intracellular survival of the bacteria and increased HLA class II surface expression in human macrophages, similar to what occurs during siRNA silencing of cathepsin S. We propose that manipulation of this miRNA might be used to enhance the innate and adaptive immune response of the host.

3.3. Materials and Methods

3.3.1. Cell lines and culture conditions

Human monocyte-derived macrophages were obtained from healthy blood donors provided by the national blood institute (Instituto Português do Sangue, Lisbon, Portugal). Differentiation of the monocytes into macrophages proceeded as previously described (Wang et al., 2010). When required, macrophages were stimulated with 100 IU/ml IFN- γ 24 h prior to infection.

3.3.2. Bacterial Cultures

The strain *Mycobacterium smegmatis* mc²155, containing a p19 (long lived) EGFP plasmid was kindly provided by Dr. Douglas Young (London School of Hygiene and Tropical Medicine, London, UK), and the green fluorescent protein (GFP)-expressing strain of *M. tuberculosis* (H37Rv-pEGFP) plasmid was a kind gift from G. R. Stewart (University of Surrey, United Kingdom). *M. smegmatis* was grown in medium containing Middlebrook's 7H9 Medium (Difco), Nutrient broth (Difco) supplemented with 0.5 % glucose and 0.05 % Tween 80 at 37 °C on a shaker at 200 r.p.m. (Anes et al., 2003). *M. tuberculosis* H37Rv was grown in Middlebrook's 7H9 medium and supplemented with 10 % OADC Enrichment (Difco) (Anes et al., 2003).

3.3.3. Infection of macrophages

Bacterial cultures on exponential grown phase were centrifuged, washed in phosphate-buffered saline (PBS). Bacteria were then resuspended in the desired culture medium without antibiotics. In order to dismantle bacterial clumps, the bacterial suspension was passed through a 21 G needle followed by 5 min ultrasonic bath. Residual clumps were removed by 1 minute centrifugation at 500 x g. Single-cell suspension was verified by fluorescence microscopy. Macrophages were infected with an MOI of 1 for 3 h at 37 °C with 5 % CO₂. Following internalization, cells were washed three times with PBS and resuspended in appropriate culture medium without antibiotics.

3.3.4. qPCR

Macrophages were seeded in 6-well plates at a density of 2 x 10⁶ cells per well. RNA was isolated and purified from infected cells using Trizol reagent (Life Technologies) and following the manufacturer protocol. miRNA expression was quantified using miRCURY LNA™ Universal RT miRNA PCR system (Exiqon) according to the manufacturer protocol and using the Exiqon LNA™ PCR primer sets: hsa-miR-106b-5p (nº 205884), hsa-miR-23a-3p (nº 204772), hsa-

miR-23b-3p (n° 204790) and hsa-miR-24-3p (n° 204260). The qPCR was performed using an ABI 7300 Real Time PCR. The reaction proceeded as follows: 1 cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1min. The miRNA expression profiles were normalized to the average obtained between miR-23a, miR-23b, and miR-24, whose expression levels were stable under the experimental conditions applied in this study (Bettencourt et al., 2013).

3.3.5. Transfection

Transfection with anti-cathepsin S siRNA or with miR-106b-5p mimics and inhibitors was performed with Biontex K2® Transfection System. Macrophages were first incubated for 2 h with 4 µl/ml of K2 Multiplier reagent in culture medium. Then, they were incubated for 24 h with the transfection reagent and 100 nM of SMARTpool ON-TARGETplus human CTSS siRNA or with miRIDIAN miRNA human hsa-miR-106b-5p mimics or hairpin inhibitors and the respective siRNA or miRNA non targeting controls (GE Dharmacon) in a ratio of 5 µl_{reagent}:1 µg_{siRNA} in antibiotic-free medium. Following that, transfection medium was removed and the cells were incubated for 3 days in fresh medium prior to any experiment in order to achieve maximum silencing (data not shown). The transfection efficiency achieved was approximately 95 %, as evaluated by flow cytometry using siGLO Green Transfection Indicator (GE Dharmacon).

3.3.6. miR-106b-5p target validation

A 413 bp fragment of the 3'-UTR of the human cathepsin S gene (CTSS) containing a sequence complementary to the seed region of miR-106b-5p, was amplified by PCR using the Phusion® Hot Start II DNA Polymerase (New England BioLabs®, MA, USA), following the manufacturer's instructions (forward primer: 5'-GCGAGCTCCAAGAAATATGAAGCACTTTCTC-3', reverse primer: 5'-CCCTCGAGTTTTTTGAAACAGAGTCTCCACT-3'). The fragment was inserted into the pmirGLO Dual Luciferase miRNA Target Expression

Vector (Promega Corporation), between the *SacI* and *XhoI* restriction sites, to originate a recombinant plasmid expressing the 3'-UTR fragment of the human cathepsin S gene. All restriction enzymes and the DNA ligase used were from New England BioLabs®. PCR products and restriction products were purified using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Recombinant plasmids were stored and propagated inside JM109 *E. coli* cells. The constructed plasmid, miR-106b-5p mimics and miRNA negative controls were transfected into HEK 293T cells using HiPerFect® Transfection Reagent (QIAGEN), following the manufacturer's instructions. After transfection, the dual luciferase assay was initiated using the Dual-Luciferase® Reporter (DLR™) Assay System kit (Promega Corporation), following all the manufacturer's instructions.

3.3.7. Western Blotting

Macrophages were seeded in 6-well plates at a density of 2×10^6 cells per well. Total proteins were recovered with 200 µl of Laemmli buffer (Sigma-Aldrich). Protein extracts were subjected to electrophoresis in 12 % SDS-PAGE gels, transferred to a nitrocellulose membrane and blocked with 0.1% Tween20, 5% of low fat milk TBS (Tris Buffered Saline). The nitrocellulose membrane was then incubated with primary antibodies specific for human cathepsin S, cathepsins B, cathepsin L and β -tubulin (abcam), overnight at 4 °C. All membranes were washed and incubated with secondary HRP-conjugated antibodies. The bands were visualized with a chemiluminescence reagent (Merck Millipore) and quantified using ImageJ (Schneider et al., 2012).

3.3.8. Flow Cytometry

Macrophages were seeded in 24-well plates at a density of 3×10^5 cells per well. Macrophages were recovered with a 5 mM EDTA/PBS solution and fixed with 4 % paraformaldehyde for 30 min. Following that, cells were stained for 30 min with antibodies specific for human HLA-DR (clone L243, Biolegend) and then analysed in Guava easyCyte™ 5HT flow cytometer.

3.3.9. Bacteria survival

Macrophages were seeded in 96-well plates at a density of 5×10^4 cells per well. When required, infected cells were lysed in 0.05 % Igepal solution. Serial dilutions of the resulting bacterial suspension were plated in Middlebrook 7H10 with 10 % OADC (Difco) and incubated for 2-3 weeks at 37 °C before colonies were observable.

3.4. Results

3.4.1. MTB induces the expression of miR-106b-5p in human macrophages

We have previously shown that MTB blocks up-regulation of several cathepsins in response to infection in macrophages via an unknown mechanism (see “Chapter 2”). Moreover, in a previously published study, we revealed the full miRNA expression profile induced by mycobacteria as well as a novel mechanism for phagocytosis regulation via MTB-dependent manipulation of a miRNA (Bettencourt et al., 2013). Therefore, we decided to search our previous results for putative miRNAs regulating cathepsins. Using target prediction tools based on the miRanda (Betel et al., 2008) and MiRtarget2 (Wang, 2008) methods we identified cathepsin S as a predicted target of miR-106b-5p. Our previous results indicated that miR-106b-5p was down-regulated early upon *M. smegmatis* infection of the mouse macrophage cell line J77A (Bettencourt et al., 2013).

To confirm that this miRNA is being regulated during infection by mycobacteria we decided to compare its expression when macrophages were infected with MTB or *M. smegmatis*. Our analysis revealed a distinct phenotype between the two species (Figure 3.1B), with *M. smegmatis* inducing a small insignificant increase in miR-106b-5p expression at 1 h and maintaining it until 24 h while MTB-infection led to an increase in miR-106b-5p expression, starting from similar levels as *M. smegmatis* and then suffering a significant 2-fold increase at 4 h which was maintained until 24 h. This demonstrates that miR-106b-5p is being specifically regulated by MTB and that this regulation is distinct from that

induced by *M. smegmatis* in primary human macrophages (Figure 3.1), or mouse macrophages (Bettencourt et al., 2013).

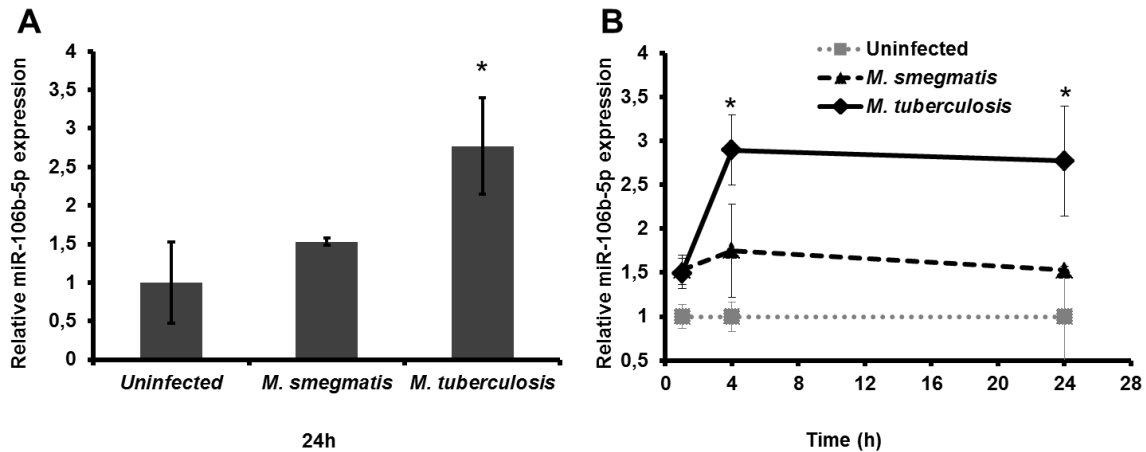


Figure 3.1. miR-106b-5p expression in macrophages infected with *M. tuberculosis* and *M. smegmatis*.

It is represented miR-106b-5p expression 24 h after infection (A) and full expression kinetics (B). Values depict gene expression relative to the uninfected control (* $p < 0.01$).

3.4.2. miR-106b-5p targets cathepsin S mRNA

Our initial results portrayed an induction of miRNA-106b-5p in MTB-infected macrophages fitting our previous findings that cathepsin S is down-regulated by MTB. To confirm this interaction we used a dual luciferase reporter vector system in which we inserted a fragment of the 3'-UTR sequence for cathepsin S into the pmirGLO dual-luciferase target expression vector. This fragment included one putative sequence complementary to the "seed region" of miR-106b-5p. Using this system we were able to assess the specific interaction between the miRNA and the 3'-UTR sequence by analyzing the resulting decrease in luciferase expression in HEK293T cells transfected with both the plasmid and the miRNA. Our results show a significant reduction in luciferase expression during co-transfection with miR-106b-5p and 3'-UTR CTSS plasmid relative to the control, thereby confirming their specific interaction (Figure 3.2A).

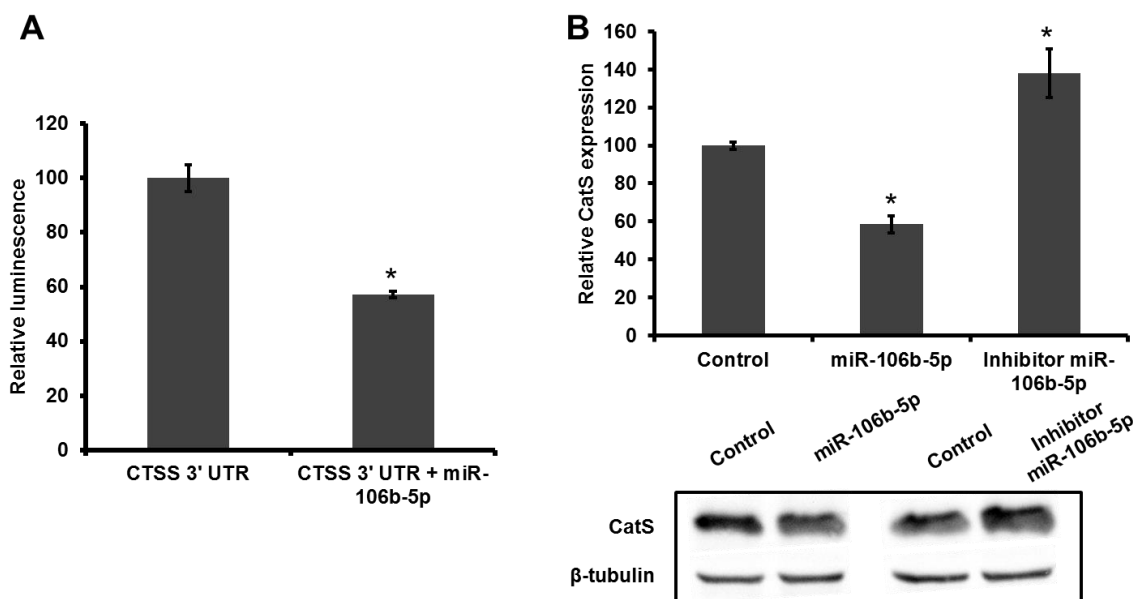


Figure 3.2. miR-106b targets cathepsin S.

In (A) luminescence was measured in HEK293T cell line transfected with CTSS 3'UTR plasmid only or in combination with miR-106b-5p mimic. Values are relative to CTSS 3'UTR-only control cells. In (B) cathepsin S protein expression in human macrophages transfected with miR-106b-5p mimics or inhibitors. Values are relative to the untransfected control (* $p < 0.001$ relative to their respective controls).

3.4.3. miR-106b-5p regulates cathepsin S expression in resting and infected macrophages

So far, our results show an early MTB-dependent up-regulation of miR-106-5p and a specific interaction between this miRNA and CatS. This correlates with our previous findings showing that MTB induces the down-regulation of CatS at 24 h, suggesting that miR-106b-5p might be tied to this regulation. To further test this hypothesis we asked if we could modulate the expression of cathepsin S by overexpressing or inhibiting the miR-106b-5p. For this, we transfected primary human macrophages with miR-106b-5p mimics or inhibitors and analyzed the protein expression of cathepsin S by western blotting. With this methodology we were able to identify up-regulation or down-regulation cathepsin S expression by manipulating mir-106b-5p (Figure 3.2). Next, we decided to test if we could produce the same results in macrophages infected with MTB. We proceeded as before, and quantified the protein expression of cathepsin S at several time points of infection (Figure 3.3). Throughout the infection we could consistently observe a reduction in cathepsin S expression in cells transfected with miR-106b-5p mimics, confirming that we could regulate

cathepsin S with this miRNA during infection. Furthermore, we were able to enhance CatS down-regulation induced by MTB after 24 h of infection.

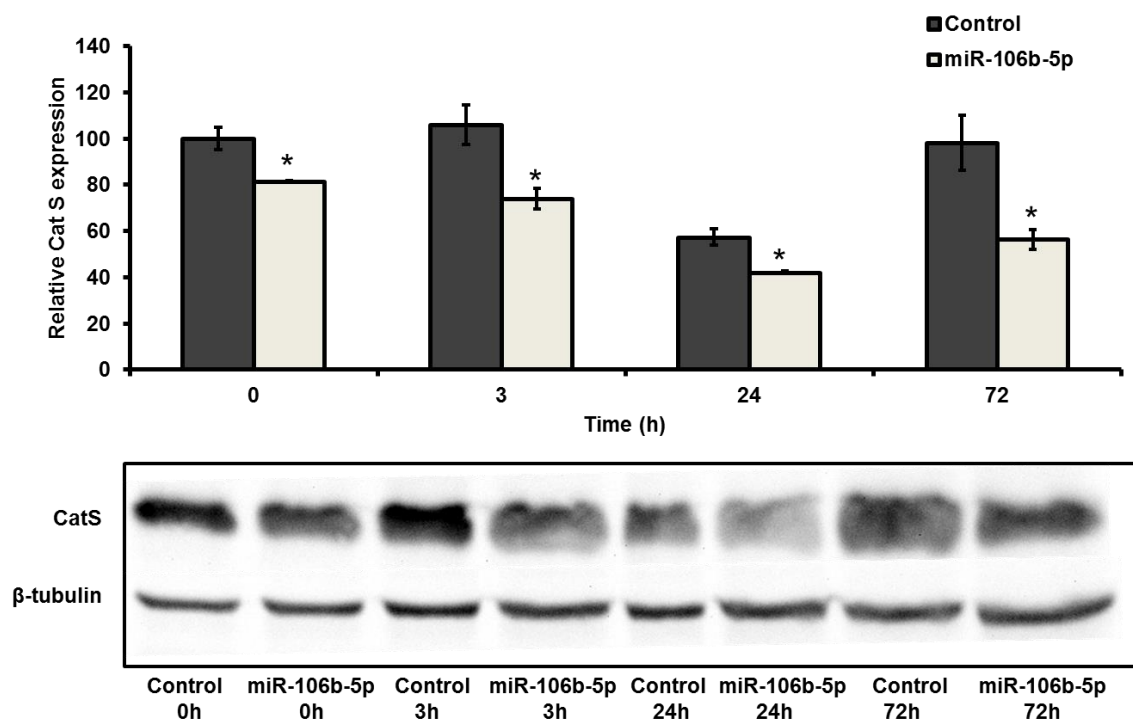


Figure 3.3. miR-106b-5p regulates cathepsin S during infection with *M. tuberculosis*. Cathepsin S protein expression in human macrophages transfected with miR-106b-5p mimics and posteriorly infected with MTB. Values are relative to the untransfected uninfected (T0) control (* $p < 0.05$ relative to the respective untransfected control).

3.4.4. miRNA-106b-5p interferes with the antigen presentation machinery

Since this miRNA is being over-expressed during MTB infection we decided to analyze the consequences of this overexpression in infected macrophages. To achieve this, we infected the transfected macrophages and searched for changes in surface expression of HLA class II complexes using flow cytometry (Figure 3.4). When we analyzed human leukocyte antigen (HLA)-DR we observed a severe increase in surface expression in cells transfected with miR-106b-5p, while with the inhibitors there was lead to no significant alterations in HLA-DR expression. To assess if this phenotype was related with a decrease in cathepsin S expression we compared the resulting effect of transducing macrophages with miR-106b-5p mimic or with siRNA specific for cathepsin S. Our results show that we were able to achieve similar results with both methods of cathepsin silencing, providing evidence that the increase in HLD-DR

expression induced by miR-106b-5p mimics is probably a result of cathepsin S down-regulation. Additionally, we tested if we could produce similar results in IFN- γ activated cells since this cytokine is reported to regulate CatS and HLA class II molecules (Beers et al., 2003, 2005; Pai et al., 2002). However, over-expression of miR-106b-5p yielded similar results in untreated or IFN- γ activated cells suggesting that HLA-DR regulation via this miRNA is independent of IFN- γ signaling pathways.

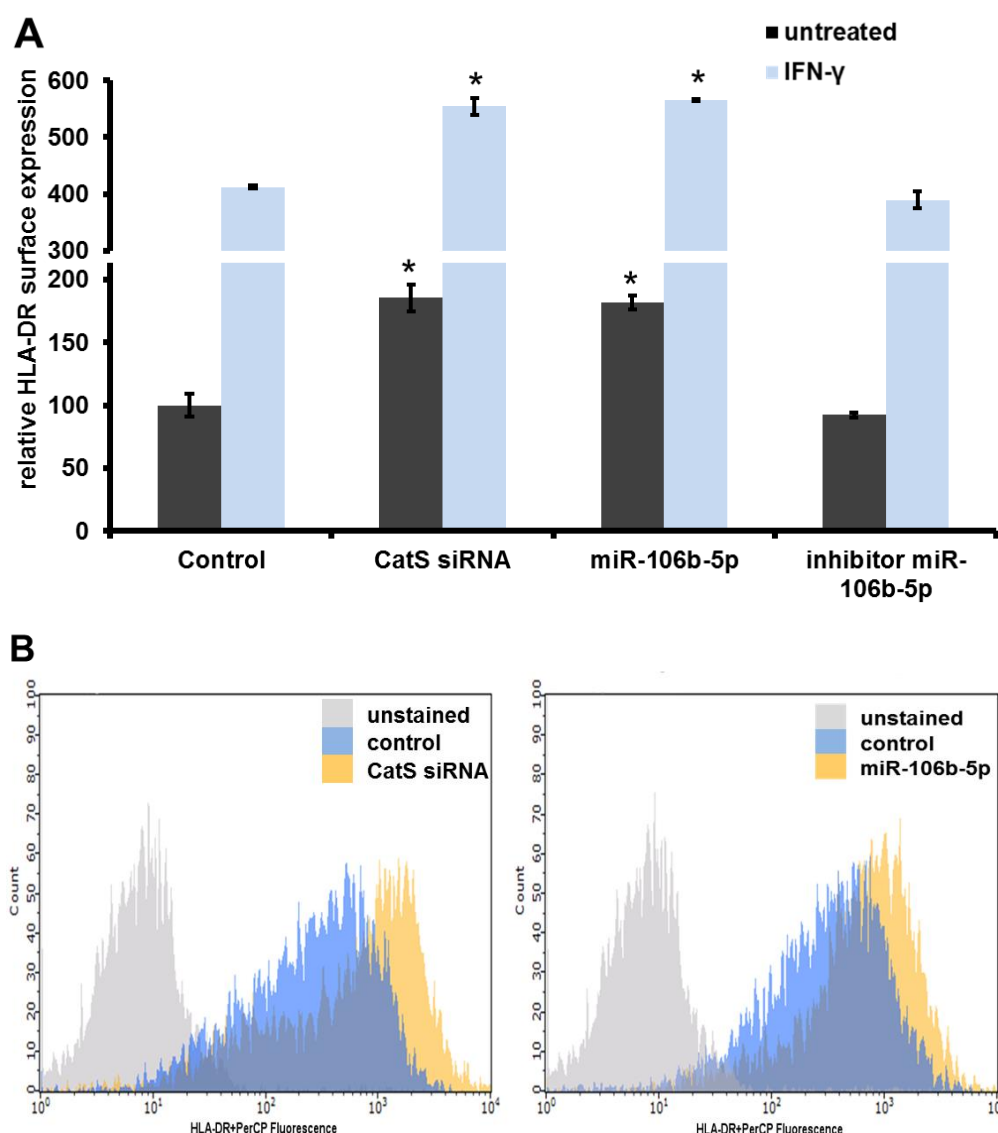


Figure 3.4. Surface expression of HLA-DR on macrophages transfected with miR-106b-5p and infected with *M. tuberculosis*.

HLA-DR surface expression was measured on macrophages infected with MTB and transfected with miR-106b-5p mimics, inhibitors or anti-cathepsin S siRNA. Values in bar plots (A) are presented as mean fluorescence intensity of the population relative to untransfected control cells (* p <0.001 relative to the respective untransfected controls). Raw values are presented in the fluorescence intensity histograms (B).

3.4.5. miR-106b-5p over-expression results in increased intracellular survival of mycobacteria

Since MTB is manipulating miR-106b-5p expression we hypothesized that this regulation will be reflected in its ability to survive inside host cells. To address this we analyzed the intracellular survival of MTB during 5 days in human macrophages transfected with the miRNA mimics or inhibitors. As expected, we could observe an increase in survival, relative to the control, that peaked at day 3 post infection (Figure 3.5A) while in macrophages transfected with the inhibitors we did not observe significant alterations (Figure 3.5B). MTB already possesses mechanisms to subvert the host so we decided to also test if we could achieve the same results using *M. smegmatis*, a species more susceptible to the killing mechanisms of the host and thus potentially benefit more from a decrease in the host antibacterial response. By executing the same experiment we could observe a significant increase in *M. smegmatis* survival in cells transfected with the miR-106b-5p mimics (Figure 3.5C), while no statistically significant differences were observed in cells transfected with the inhibitors (Figure 3.5D). Next, we expected that since miR-106b-5p targets cathepsin S, we should observe a similar increase in MTB survival when we silence cathepsin S expression using siRNAs. Comparing the effects of transfecting cells with miR-106b-5p mimics and cathepsin S siRNAs we could produce similar results in MTB intracellular survival (Figure 3.5E) and thus we validate the interaction between miR-106b-5p and cathepsin S.

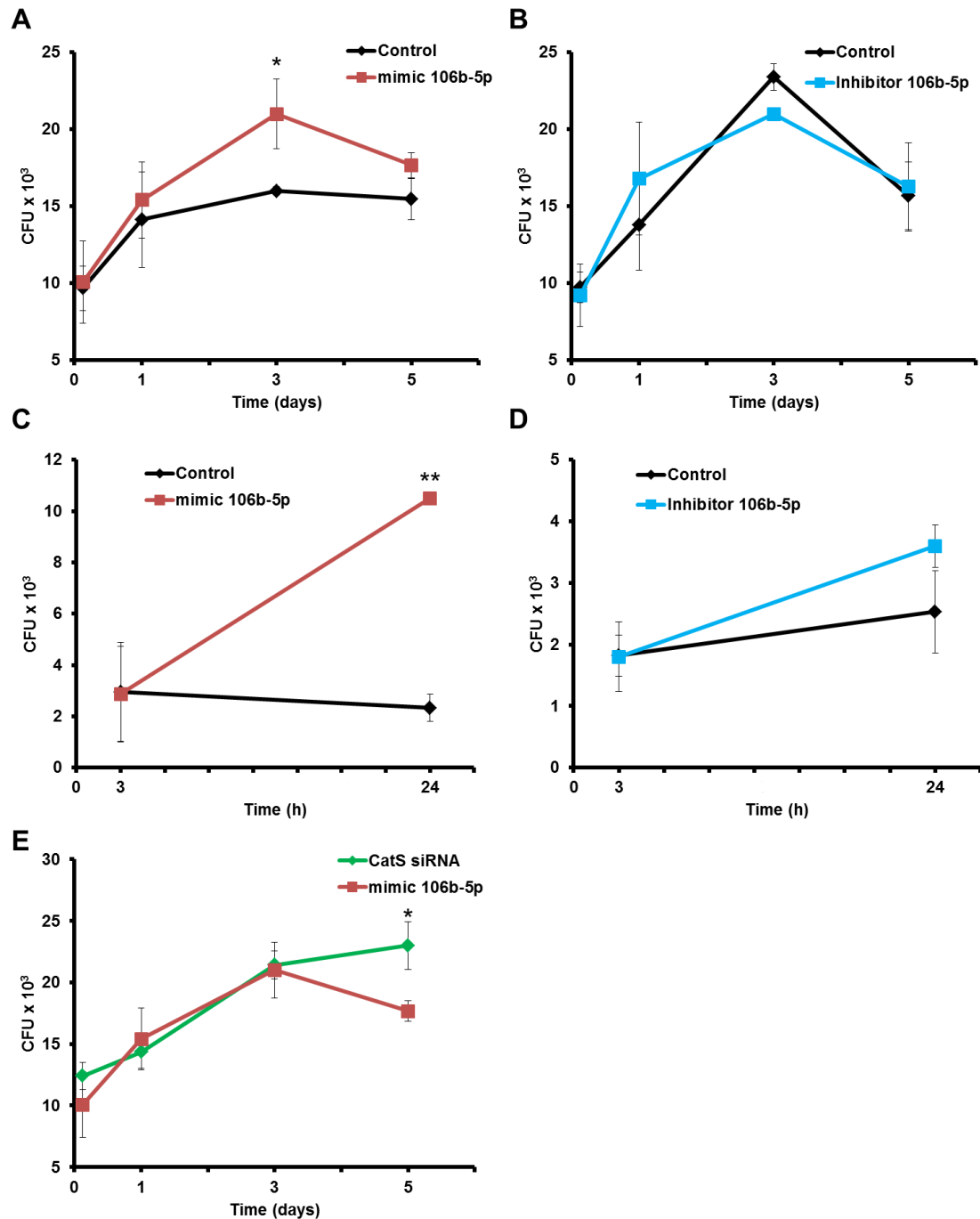


Figure 3.5. Intracellular survival of mycobacteria in macrophages transfected with miR-106b-5p. Bacterial intracellular survival was measured in macrophages transfected with miR-106b-5p mimics or inhibitors and infected with MTB (A and B) or with *M. smegmatis* (C and D) or transfected with miR-106b-5p mimics or CatS siRNA and infected with MTB (E). Values depict mean colony-forming units (*p<0.5; **p<0.01).

3.5. Discussion

Our recent work implicated miRNAs in the control of the internalization stage of phagocytosis during infection with mycobacteria (Bettencourt et al., 2013). *M. tuberculosis* has been found to exploit this mechanism of gene regulation in order to impair the detrimental mechanisms of host macrophages and improve its survival. Pathogenic mycobacteria manipulate miRNAs to inhibit caspase-dependent apoptosis (Fink, 2005), pro-inflammatory cytokine production (Ma et al., 2011; Rajaram et al., 2011; Tili et al., 2007) and TLR-mediated signaling (Liu et al., 2009, 2012).

Our results indicate that another miRNA, miR-106b-5p modulates lysosomal proteolysis by regulating cathepsin S expression. Cathepsin S is predominantly expressed in antigen presenting cells such as macrophages and DCs (Hsing and Rudensky, 2005) and is a major protease in antigen presentation by processing HLA class II molecules (Rudensky and Beers, 2005). Here we show that MTB-infection results in a decreased expression of Cat S in macrophages and DCs comparatively with *M. smegmatis*. In addition, this down-regulation improves MTB intracellular survival and also increases surface expression of HLA class II molecules. This effect was most prominent after 24 h of infection, although an increase in miR-106b-5p expression was already detected early at 4 h of infection. This period between miRNA up-regulation and Cat S decreased protein expression is probably related with the half-life of Cat S reported to be approximately 16-18 h (Nissler et al., 1999). Other mechanisms have been proposed for Cat S regulation involving post-translational mechanisms controlling Cat S activity, such as pH regulation or cystatin-mediated inhibition.

Macrophage activation by IFN- γ is reported to induce a strong oxidative response which increases the phagosomal pH and results in lower proteolytic activity (Yates et al., 2007) possibly due to the instability of acidic cathepsins at that range of pH values. However Cat S is an exception since it is stable and retains its activity at neutral or slightly alkaline pH (Kirschke et al., 1989). Moreover, this activation results in the down-regulation of cystatin C, a major inhibitor of CatS activity (Pierre and Mellman, 1998; Zavasnik-Bergant et al., 2005). Accordingly, when we activated macrophages with IFN- γ , the surface

expression of HLA-DR suffered a severe increase. Yet macrophages transfected with miR-106b-5p developed the same phenotype and were able to enhance HLA-DR surface expression as well as in non-activated macrophages, suggesting this regulation is independent of IFN- γ . This might also argue against another IFN- γ -induced mechanism of HLA class II regulation (Pai et al., 2002), dependent on an indirect inducer of HLA class II transcription, the class II transactivator (CIITA) (Boss and Jensen, 2003). Another study described a different mechanism for CatS regulation involving the induction of IL-10 secretion via infection with *M. bovis* BCG (BCG) (Sendide et al., 2005). These authors proposed that BCG regulates CatS by inhibiting IFN- γ -dependent activation of CatS and by inducing the secretion of IL-10 in infected macrophages. More recently others have also shown that induction of IL-10 results in decreased CatS and HLA class II expression (Chan et al., 2010).

Although in our previous studies we have observed that MTB inhibits HLA-DR surface expression and CatS expression, relative to the infection with *M. smegmatis*, our results disproved any causal relation between those events (see “Chapter 2”). On the contrary, our proposed mechanism via induction of miR-106b-5p resulted in a similar outcome in mycobacteria survival and antigen presentation, to that of CatS siRNA silencing. This provides a strong evidence for direct regulation of CatS by the manipulation of a miRNA by MTB. We cannot rule out the possibility that miR-106b-5p is regulating other genes. Indeed, miRNAs are known to target several genes, resulting in complex regulatory networks. Nonetheless, our results indicate a strong impact of miR-106b-5p in macrophage-mediated response to MTB. Our study opens the door for future manipulation of miR-106b-5p to enhance the anti-microbial activity of innate immune cells. Future studies should focus on better characterizing the mycobacterial effectors leading to this regulation and also on the development of methods to counter it. This miRNA might also serve as an additional biomarker of tuberculosis. Several studies have also focused on the relevance of miRNAs as biomarkers of disease since they were found in circulating PBMCs (Liu et al., 2011; Wu et al., 2012), serum (Fu et al., 2011) and sputum (Yi et al., 2012) of tuberculosis patients. Additionally, CatS is an established relevant enzyme in cancer and other pathologies (Fonović and Turk, 2014b;

Mohamed and Sloane, 2006). Further studies of this miRNA might also prove to be useful for those diverse fields of biomedical research.

3.6. Acknowledgments

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Chapter 4: Ion channel blockers act as antimicrobial agents and enhancers of macrophage killing activity against drug resistant *Mycobacterium tuberculosis*

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In this chapter, I contributed in the design, execution and analysis of all the macrophage/bacteria interaction experiments, the ATP and nitric oxide experiments in bacteria and I contributed to the writing of the manuscript.

Ion channel blockers act as antimicrobial agents and enhancers of macrophage killing activity against drug resistant *Mycobacterium tuberculosis*

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4.1. Abstract

Here we investigate the antimycobacterial properties of ion channel blockers verapamil, thioridazine, chlorpromazine, flupenthixol and haloperidol, by deciphering their inhibitory effects directly in *M. tuberculosis in vitro* and in infected human monocyte-derived macrophages. *In vitro*, all compounds exhibited synergistic inhibitory activities when combined with the main first line drugs, isoniazid and rifampicin, and were able to inhibit active efflux. Gene expression analysis of the bacteria showed that efflux genes were overexpressed in response to antibiotic exposure, *in vitro* and *ex vivo*, indicating that *M. tuberculosis* drug resistance within macrophages is also mediated by the expression of efflux pumps. The compounds displayed a rapid and high killing activity for *M. tuberculosis*, correlated with a decrease in bacterial ATP levels. We show that the compounds lead to a significant decrease in the intracellular mycobacterial load, consequence of ion channel blocking at the level of endocytic membranes leading to phagosome acidification and activation of lysosomal hydrolases. Altogether, we demonstrate that these inhibitors display a dual antimycobacterial inhibitory effect as they target both the bacteria and the host cell. Disruption of the proton motive force (PMF), due to inhibition of the respiratory chain, results in the inhibition of *M. tuberculosis* PMF dependent efflux systems, therefor promoting the retention of antibiotics subject to active efflux. Concerning the host cell side, the phagosomal acidification stimulated by these compounds synergize with several components of the host immune response, restricting *M. tuberculosis* intracellular growth. In conclusion, we provide direct, experimental evidence that these ion channel blockers act as

antimicrobial agents and enhancers of macrophage killing activity. This work further highlights the value of these compounds as adjuvants of drug resistant tuberculosis chemotherapy.

4.2. Introduction

Mycobacterium tuberculosis is a challenging pathogen, mostly due to its impenetrable cell wall coupled with a long generation time, a plastic metabolism and a remarkable ability to establish persistent and opportunistic infections. Consequently, the therapeutic regimen for tuberculosis requires a prolonged antibiotic treatment of, at least, 6 months in order to attain a favourable clinical outcome (Mitchison and Davies, 2012). Although infections with drug-susceptible *M. tuberculosis* (MTB) strains can be effectively treated with the current regimen of first-line antituberculosis drugs, the increase of antibiotic resistant MTB strains represents an ongoing threat to the control of the disease. According to the World Health Organization, in 2012, there were an estimated 450 000 new cases of multidrug resistant tuberculosis (MDRTB, simultaneous resistance to isoniazid and rifampicin) worldwide. The average proportion of MDRTB cases with extensively drug resistance (XDRTB, MDR plus resistance to quinolones, aminoglycosides and cyclic peptides) was 9.6% (WHO, 2013a). Regrettably, these numbers indicate that, despite all efforts, antibiotic resistance in tuberculosis continues to increase.

Resistance to antituberculosis drugs are assumed to be a consequence of the development of spontaneous mutations in defined genes or its promoter regions and the resulting resistance levels can be influenced by the type of alteration in the target gene (Böttger, 2011). However, in a proportion of strains, the resistance to a certain drug cannot be solely explained by classical gene mutations, which suggests the presence of additional mechanisms of resistance (Louw et al., 2011; Machado et al., 2012). In mycobacteria, the physiological resistance is generally attributed to the low permeability of the cell wall, which may limit the entry of antibiotics. Besides this reduced permeability, efflux systems also promote the development of resistance by the extrusion of

molecules entering the cell prior to their access to the intended target (Louw et al., 2009).

Previous studies have stated the contribution of efflux mechanisms to antibiotic resistance in MTB describing several putative efflux pumps of different classes shown to be involved in the transport of different compounds (Viveiros et al., 2012). Furthermore, it has been demonstrated that the overexpression of efflux pumps contributes to drug-resistance by allowing the bacteria to survive for a longer period of time in the presence of sub-inhibitory concentrations of antibiotics, until mutations emerge (Machado et al., 2012). Concomitantly, it was demonstrated that the resistance level to a given antibiotic can be significantly reduced by an efflux inhibitor despite the presence of a mutation, highlighting the contribution of active efflux to the overall resistance level of a drug resistant strain of MTB (Louw et al., 2011; Machado et al., 2012).

The combination of an efflux inhibitor to with tuberculosis chemotherapy can enhance antimycobacterial killing, prevent the emergence of drug resistance (Adams et al., 2011; Machado et al., 2012; Rodrigues et al., 2012) and reduce the duration of tuberculosis treatment (Adams et al., 2011; Gupta et al., 2013). To date, several compounds have been appointed as potential efflux inhibitors. These compounds are ion channel blockers and comprised the phenothiazines thioridazine (TZ), chlorpromazine (CPZ) and its derivative flupenthixol (FPX) a high-potency thioxanthene; haloperidol (HAL) a butyrophenone structurally and clinically related with phenothiazines; verapamil (VP), a phenylalkylamine (Viveiros et al., 2012). These compounds are FDA approved drugs, used to treat several mental disorders or hypertension, with well-characterized pharmacology and toxicology profiles.

Ion channel blockers such as TZ and VP have been shown to actively inhibit efflux and *in vitro* growth of MTB strains, alone or in combination with antimycobacterial drugs (Amaral et al., 1996, 2007; Machado et al., 2012; Silva et al., 2001). TZ demonstrated significant activity against MDRTB in a murine model (van Soolingen et al., 2010) and it has been successfully employed to treat XDRTB patients on the basis of compassionate reasons (Abbate et al., 2012). VP has been shown to be the most potent mycobacterial inhibitor to

date, being able to enhance the inhibitory activity of isoniazid (Machado et al., 2012; Rodrigues et al., 2012) and rifampicin (Louw et al., 2011) in MTB clinical strains. Recently, Gupta et al. (2014) demonstrated that efflux inhibition by VP can potentiate the activity of bedaquiline, a diarylquinoline recently approved for the treatment of MDR-TB (WHO, 2013b). It has also been shown that the addition of VP accelerates the bactericidal and sterilising activities of tuberculosis therapy in the mouse model (Gupta et al., 2013). Beyond their antimycobacterial activity, these compounds also present immunomodulatory abilities on MTB-infected macrophages. Previously, we had shown that inhibitors of calcium and potassium can enhance the killing of MTB by macrophages (Martins et al., 2008; Ordway et al., 2003). Afterwards, it was demonstrated that the inhibition of macrophage calcium channels increases calcium influx, which, consequently, activates an immune response against intracellular MTB (Gupta et al., 2009). Further evidence in favour of the usefulness of the ion channel blockers is reported in the elegant studies by Adams *et al.* (2011, 2014), wherein the selective pressure exerted by the macrophage on internalized MTB can induce the bacteria efflux pumps and thereby drug-tolerance. Additionally, the authors also show that this macrophage drug-induced tolerance can be reduced by VP or derived metabolites (Adams et al., 2011, 2014).

Having established the significance of the use of ion channel blockers as adjuvants in tuberculosis chemotherapy, it is important to understand their antimycobacterial properties both *in vitro* and in the macrophage model. In this study, we demonstrate that these compounds are efflux inhibitors with direct antimycobacterial activity towards MTB. We show that they can enhance the inhibitory activities of the antibiotics against MTB *in vitro* and enhance the killing activity of the macrophage against intracellular MTB. Furthermore, we demonstrate that these compounds are able to induce phagosome acidification, whose combined effect with other antimicrobial macrophage factors, promote the elimination of the internalized mycobacteria. Our findings contribute to a better understanding of the mode of action of these ion channel blockers and provide insights to identify the mediators of their antimycobacterial and immunomodulatory activity.

4.3. Materials and Methods

4.3.1. Mycobacterial strains and growth conditions.

A panel of nine *M. tuberculosis* strains were selected for this study: *M. tuberculosis* H37Rv ATCC27294^T, *M. tuberculosis* H37Rv Δ *katG* (Machado et al., 2012) and seven drug-resistant clinical strains obtained from the culture collection of the Mycobacteriology laboratory of the Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (Table 4.1). *M. bovis* BCG Pasteur ATCC35734 expressing green fluorescent protein (BCG-GFP) was kindly provided by Prof. M. Niederweis (University of Alabama). Cultures of *M. tuberculosis* were routinely grown in MGIT media (Becton Dickinson, Diagnostic Systems, Sparks, MD, USA) supplemented with 10% OADC (oleic acid/albumin/dextrose/catalase) (Becton-Dickinson), except when indicated otherwise. Susceptibility testing, semi-quantitative drug susceptibility testing and minimum inhibitory concentration determinations (MIC) were conducted using the BACTECTM MGITTM 960 system (MGIT960).

4.3.2. Antibiotics and chemicals.

Isoniazid, rifampicin, ofloxacin, amikacin, capreomycin, verapamil (VP), flupenthixol (FPX), thioridazine (TZ), chlorpromazine (CPZ), haloperidol (HAL), and the efflux substrate ethidium bromide (EtBr), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were prepared in sterile deionized water except rifampicin and haloperidol which were prepared in dimethyl sulfoxide (DMSO). The lyophilized drugs (BACTECTM MGITTM 960 SIRE and PZA kits; SIRE: streptomycin, isoniazid, rifampicin and ethambutol; PZA: pyrazinamide) used in standard susceptibility testing were purchased from Becton Dickinson and the stock solutions prepared as per manufacturer's instructions.

4.3.3. Susceptibility testing.

(i) First and second line drug susceptibility testing.

The MGIT960 was used for first and second line drug susceptibility testing according to the manufacturer's instructions. MGIT tubes were inoculated with 0.8 ml SIRE supplement (Becton Dickinson), 0.1 ml of antibiotic at the critical concentrations (0.1 µg/ml for isoniazid, 1 µg/ml for rifampicin, 1 µg/ml for streptomycin, 5 µg/ml for ethambutol, 100 µg/ml for pyrazinamide, 1 µg/ml for amikacin, 2.5 µg/ml for capreomycin, and 1 µg/ml for ofloxacin) and 0.5 ml of the suspension of the strain. For preparation of the drug-free proportional control strain suspension was diluted 1:100 (1:10 for PZA) and 0.5 ml inoculated in the tube. The results were interpreted as follows: at the time of positivity of the proportional control (Growth units [GU] = 400), the comparison between this tube and the tubes containing the drugs(s) was performed. If the GU of the tubes containing the drug were >100, they were considered to be resistant to that concentration. If the GU of the tube containing the drug was <100 they were considered susceptible (Springer et al., 2009). Growth of the cultures was monitored with the Epicenter V5.80A software equipped with the TB eXIST module.

(ii) MIC determination of antibiotics and inhibitors.

MIC determination was done within MGIT960 and the growth monitored with the Epicenter V5.80A software. Verapamil and haloperidol were tested at concentrations ranging from 16 to 512 µg/ml; thioridazine, chlorpromazine and flupenthixol were tested at concentrations from 3.75 to 60 µg/ml. Isoniazid was tested a concentration ranging from 0.1 to 120 µg/ml, rifampicin and amikacin were tested from 1 to 640 µg/ml and ofloxacin was tested from 1 to 10 µg/ml. At the time of testing, two-fold serial dilutions were prepared to achieve the desired concentrations. The MIC was considered as the lowest concentration with GU <100 when the drug-free control tube reached a GU value of 400.

(iii) Quantitative drug susceptibility testing of antibiotics in presence and absence of inhibitors.

Quantitative drug susceptibility (qDST) testing of rifampicin, isoniazid, ofloxacin and amikacin was conducted using the MGIT960 and the Epicenter V5.80A/TB

eXIST. Isoniazid was tested at 0.1, 0.4, 1, 3 and 10 µg/ml, rifampicin and amikacin at 1, 4 and 20 µg/ml, and ofloxacin at 1, 2 and 10 µg/ml. The interpretation of the results was performed as per Springer et al. (2009). For the quantification of the resistance levels of each strain, the following criteria was used: isoniazid low-level resistance when resistant (R) at 0.1 and susceptible (S) at 1 µg/ml; isoniazid high-level resistance when $R \geq 1$; rifampicin and amikacin low-level resistance when R at 4 and S at 20 µg/ml; rifampicin and amikacin high-level resistance when $R \geq 20$ µg/ml; ofloxacin low-level resistance when R at 1 and S at 2 µg/ml; ofloxacin high-level resistance when $R \geq 2$ µg/ml. The inhibitors were used at $\frac{1}{2}$ of the respective MIC.

(iv) Determination of the synergistic effect of the inhibitors.

The analysis of the effect of drug combination was achieved by calculating the fractional inhibitory concentration (FIC) index as follows: $FIC = \frac{MIC_{ATB \text{ in combination}}}{MIC_{ATB \text{ alone}}}$. The FIC was interpreted as: $FIC \leq 0.5$, synergism; $>0.5-1$, indifference; and >1 , antagonism (Lorian, 1996).

4.3.4. Time-kill kinetics.

To assess the bactericidal activity of the inhibitors, time-kill studies were performed with two strains: the reference strain H37Rv (drug-susceptible) and one clinical strain, 149/09 (drug-resistant with XDR pattern – see Table 4.1). Mid-log phase cultures were diluted to 1×10^5 CFU/ml and challenged with the compounds alone or in combination with antibiotics. Strains were inoculated in 100 ml of MB7H9 (DIFCO, Madrid, Spain) containing 10 % OADC enrichment, 0.05 % Tween 80 and the compounds at five times the MIC. A drug-free control was included in the assay to monitor the normal growth of the strains. Cultures were sampled for CFU determination after 1, 2, 3 and 7 days of incubation at 37°C. For CFU determination, the samples were pelleted once by centrifugation at $16060 \times g$ for 10 minutes to reduce drug carry-over and resuspended in drug-free MB7H9. Colony forming units' were determined with the MGIT960 and the Epicenter software. Briefly, to generate calibration curves, the number of viable cells was first determined by standard plate counting method. Cell suspensions were grown to an optical density at 600 nm (OD_{600}) of 0.8 and 1 ml was added

to 9 ml of MB7H9. Tenfold serial dilutions (10^0 to 10^{-7}) were made in MB7H9 and 100 μ l from each dilution plated onto MB7H11 plates (DIFCO) supplemented with 10 % OADC. Plates were sealed and the CFU's determined after incubation at 37°C during 21 days. Simultaneously, MGIT tubes supplemented with 10 % OADC were inoculated in triplicate with 100 μ l of the same serial dilutions, incubated in the MGIT960 system and the TTD recorded. Calibration growth curves for each dilution were generated with the Epicenter software and the TTD for each dilution plotted against \log_{10} CFU. To obtain the cell concentration for each drug exposed culture, CFU's were predicted by the theoretical \log_{10} CFU based TTD multiplied by the corresponding dilution factor. The killing effect of the compounds on *in vitro* *M. tuberculosis* growth was defined as the lack of growth in MGIT tubes after 100 days of incubation (Filippini et al., 2010).

4.3.5. Quantification of intracellular ATP

Intracellular ATP was quantified by using the ATP Determination Kit (Invitrogen, Life Technologies, Paisley, UK) according to the manufacturer's instructions. Briefly, strains were exposed to the compounds and at various time points, aliquots of 1 ml of bacteria were collected, heat inactivated and immediately deep frozen. Cell lysates were transferred into white flat bottom 96-well plates and the ATP content measured using a Tecan's Infinite® M200 plate reader (Tecan Trading AG, Switzerland) and expressed as relative luminescence units. ATP standards were used in all experiments as internal controls. Sample ATP levels were calculated from the calibration curve, with correction for background luminescence. Isoniazid and rifampicin were used as controls. Cultures were sampled for CFU determination as described above.

4.3.6. Growth rate measurements

Cell suspensions were adjusted to 150 GU with sterile saline solution and 0.5 ml transferred into a MGIT tube containing 10 % OADC. Growth rates were measured at 37 °C with the MGIT960 and the Epicenter software. First, we

determined the time to detection, *i.e.* the time to reach the threshold of 75 GU, as a measure of the capacity of the strain to adapt to a new environment. The relative growth rates were determined according to Gullberg et al. (2011) with modifications. The calculations were based on TTD values between 75 and 400 GU which corresponds to OD₆₀₀ values between ≈ 0.02 and 0.1 (data not shown). The relative growth rates were determined as the ratio between the growth rate of each strain and the growth rate of the control strain. Data was normalized against the control strain *M. tuberculosis* H37Rv.

4.3.7. Mutation frequency determinations

M. tuberculosis H37Rv was grown in 200 ml erlenmeyer flasks supplemented with 10 % OADC and 0.05 % Tween 80 and incubated at 37 °C until they reach an OD₆₀₀ of 0.8-0.9. Cultures were diluted to 1×10^4 CFU/ml in MB7H9. Spontaneous mutants resistant to the inhibitors were detected by plating 0.1 ml of this dilution onto MB7H11 agar plates containing the compounds at concentrations five times the MIC. Plates were sealed in plastic bags and the colonies counted after three weeks of incubation at 37 °C. The mutation frequency corresponds to the ratio of the average number of mutants per milliliter to the total number of cells per milliliter (David, 1970).

4.3.8. Detection of catalase activity

Catalase activity was assessed according to the protocol described by David (1989), at room temperature. The *M. tuberculosis* H37Rv strain served as a positive control.

4.3.9. Acid resistance evaluation

M. tuberculosis H37Rv was grown in 25 ml of MB7H9 containing 10 % OADC and 0.05 % Tween 80 until they reach an OD₆₀₀ of 0.8. Cells were washed by centrifugation at 2940 x *g* during 3 minutes, to remove Tween 80. Washed cells

were diluted to 1×10^5 CFU/ml in 100 ml of MB7H9 at different pH's ranging from 6.8 to 3.5 plus 10 % OADC without Tween 80 (Vandal et al., 2008). Cultures were sampled for CFU determination after 1, 2, 3 and 7 days of incubation at 37 °C. For CFU determination, samples were washed once by centrifugation at $16060 \times g$ for 10 minutes and resuspended in MB7H9. Colony forming units were determined with the MGIT960 and the Epicenter software as described above.

4.3.10. Antimycobacterial activity on infected macrophages

(i) Mycobacterial cultures.

M. tuberculosis strains and *M. bovis* BCG-GFP were cultured in Middlebrook 7H9 broth supplemented with 10 % OADC and 0.05 % tyloxapol (Sigma-Aldrich) at 37 °C, with occasional shaking. For BCG-GFP, 50 µg/ml hygromycin (Sigma-Aldrich) were added to the media. Prior to any experiment, bacteria were prepared in order to achieve a single cell suspension. Bacterial cultures on exponential growth phase were centrifuged at $3000 \times g$ for 10 minutes, washed in PBS and resuspended in cell culture medium without antibiotics. In order to disrupt bacterial clumps, the cell suspension was passed through a 21 G needle. Residual clumps were removed by 1 minute centrifugation at $500 \times g$.

(ii) Isolation and culture of human monocyte-derived macrophages.

Human monocyte-derived macrophages (HMDMs) were obtained from buffy coat preparations kindly donated by Instituto Português do Sangue. The buffy coat was diluted (1:1) in PBS containing 0.5 % bovine serum albumin (BSA) and 2 mM EDTA and then gently overlaid on Ficoll-Paque Plus (GE Healthcare, Freiburg, Germany) at a ratio of 2:1 followed by $800 \times g$ centrifugation for 20 minutes at room temperature. The interface was then recovered and washed two times. Selection of CD14 monocytes was performed using MACS LS cell separation system (MiltenyiBiotec, Cologne, Germany) according to the manufacturer instructions. Briefly, cells were incubated during 30 minutes at 4 °C with anti-CD14 MicroBeads (MiltenyiBiotec) and then passed through a magnetic separator. CD14 monocytes were recovered and differentiated into macrophages during 7 days in macrophage medium containing RPMI-1640

medium with 10 % fetal calf serum (FCS), 1 % GlutaMAX™, 1 mM sodium pyruvate, 10 mM HEPES at pH 7.4, 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies), and 20 ng/ml M-CSF (Immunotools, Friesoythe, Germany) and incubated at 37 °C in 5 % CO₂ atmosphere. Fresh medium was added at day 4 post isolation.

(iii) Determination of macrophage viability after treatment with compounds.

HMDMs were seeded 3x10⁵ cells per well in 24-well plates and treated with the compounds. After 3 days of treatment, cell viability was determined using AlamarBlue (Molecular Probes, Life Technologies) following the manufacturer's indications. Briefly, 10 % AlamarBlue reagent was added to each well and incubated for 4 hours at 37 °C and 5 % CO₂. Then, reduction of resazurin to resorufin was measured, through the fluorescence emission, at an excitation of 570 nm and emission of 595 nm in a Tecan's Infinite M200 plate spectrophotometer. For the subsequent intracellular assays, the compounds were used at concentrations that were shown to be non-toxic to the macrophages. Isoniazid was used at critical concentration of 0.1 µg/ml.

(iv) Quantification of intracellular bacterial survival.

HMDMs were infected with *M. tuberculosis* strains at MOI 1 and were allowed to uptake the bacteria for 3 hours. Next, they were washed three times with PBS and maintained in macrophage medium without antibiotics. At 3 hours, and 1, 3, 5 and 7 days post-infection, cells were lysed with 0.05 % Igepal (Sigma-Aldrich) solution in water. Serial dilutions of the lysate were plated on MB7H11 medium supplemented with 10 % OADC. Colony forming units were counted upon 3 weeks of incubation at 37 °C. The capacity of intracellular growth of each strain was calculated as the ratio between the CFU during the 5 day protocol and the CFU on day 0 (Rey-Jurado et al., 2011).

(v) Quantification of compound-induced macrophage acidification.

HMDMs were infected with BCG-GFP at MOI 10 as described above. At 24 hours post-infection, cells to be analysed by fluorescence microscopy were incubated with 100 nM LysoTracker Red for 2 hours or 1 µM LysoSensor™ Green (Molecular Probes) for 10 minutes and then washed and observed under a fluorescence microscope. Quantification of acidified cells by flow cytometry

was performed by detaching the cells with 0.05 % Trypsin-EDTA (Sigma-Aldrich). After cells were washed with PBS and further incubated with 100 nM LysoTracker Red (Molecular Probes) for 2 hours at 37 °C and 5 % CO₂. Cells were washed and analyzed on an easyCyte™ 5HT flow cytometer (Millipore Corporation, Billerica, MA, USA).

(vi) *Measurement of cathepsin B activity.*

HMDMs were infected with *M. tuberculosis* H37Rv at MOI 1 for 3 hours as described above. The cells were then incubated with the compounds during 24 hours at 37 °C with 5 % CO₂. Cathepsin B activity was measured using a fluorometric cathepsin B activity assay kit (BioVision, California, USA) in accordance with the manufacturer's instructions. After 24 hours of infection the cells were harvested, lysed in chilled cathepsin B cell lysis buffer for 10 minutes and mixed with equal volume of reaction buffer. The suspensions were transferred to black flat bottom 96-well plates and incubated with 200 µM cathepsin B substrate (Ac-RR-AFC) during 2 hours at 37 °C. Cathepsin B activity was measured at 400 nm excitation and 505 nm emission using a Tecan's Infinite® M200 plate reader and expressed as relative fluorescence units (RFU).

4.3.11. Assessment of efflux activity.

The detection of ethidium bromide efflux was performed by a semi-automated fluorometric method using a Rotor-Gene 3000™ thermocycler (Corbett Research, Sidney, Australia) (Viveiros et al., 2010) with a modified protocol for *M. tuberculosis* (Machado et al., 2012). The semi-automated fluorometric method was applied to all strains. Briefly, the strains were grown in 10 ml of MB7H9 medium containing 10 % OADC enrichment (Becton Dickinson) and 0.05 % Tween 80. Cultures were incubated at 37°C, without stirring, until they reached an OD₆₀₀ of 0.8. After the cultures reached the desired OD₆₀₀, cultures were centrifuged at 2940 x g, during 3 minutes at 25 °C. After this, the supernatant were discarded, the pellet washed, resuspended in PBS and centrifuged as before.

(i) Accumulation of ethidium bromide.

For the ethidium bromide accumulation assays, the washed cells were resuspended in PBS and the OD₆₀₀ adjusted to 0.8. In order to determine the lowest concentration of ethidium bromide that causes accumulation, 50 µl of the bacterial suspension was added to 0.2 ml PCR tubes containing different concentrations of ethidium bromide that ranged from 0.125 to 5 µg/ml and glucose at a final concentration of 0.4 %. The final OD₆₀₀ of the bacterial suspension in the assay was 0.4. The assays were conducted at 37 °C in a Rotor-Gene 3000TM, and the fluorescence of ethidium bromide was measured (530/585 nm) at the end of each cycle of 60 seconds, for 60 minutes. After determining the higher concentration of ethidium bromide that do not causes accumulation, the effect of the inhibitors on the accumulation of ethidium bromide was evaluated. These assays were performed like described above with each efflux inhibitor at ½ of the MIC (a subinhibitory concentration that do not affect bacterial viability but inhibits efflux), ethidium bromide at the higher concentration that do not cause accumulation (determined for each strain), 37 °C and with and without glucose. To better evaluate the effect of the inhibitors in the accumulation of ethidium bromide, for each assay we determined the relative final fluorescence (RFF) at the last time point (minute 60) of the assay in comparison with the control condition using the formula $RFF = (RF_{treated} - RF_{non-treated}) / (RF_{non-treated})$ (Machado et al., 2011). Here, $RF_{treated}$ corresponds to the relative fluorescence for each strain treated with the compound at the last time point of the accumulation assay and the $RF_{non-treated}$ corresponds to the relative fluorescence of the cells only in the presence of ethidium bromide at the same time point. High RFF values indicated that cells accumulate more ethidium bromide under the condition used than those of the control (non-treated cells). Negative RFF values indicated that treated cells accumulated less ethidium bromide than those of the control condition. Each assay was performed in triplicate and the results presented correspond to the average ±SD of three independent assays.

(ii) Efflux of ethidium bromide.

For the efflux assays, the washed cells were resuspended in PBS and the OD₆₀₀ adjusted to 0.4. The ethidium bromide loaded cells were prepared by

incubating the bacterial suspension, in glass test tubes, with the higher concentration of ethidium bromide that do not cause accumulation (determined above), under conditions that promote accumulation: the most effective efflux inhibitor at $\frac{1}{2}$ of the MIC, absence of glucose at 25 °C during 60 minutes. For all strains tested in this work, the most effective inhibitor was verapamil. After ethidium bromide accumulation, the cells were collected by centrifugation at 4860 x g during 5 minutes at 25 °C and resuspended in PBS to an OD₆₀₀ of 0.8. After adjusting de OD₆₀₀, 50 µl of the bacterial suspension was added to 0.2 ml PCR tubes containing (1) PBS only; (2) PBS plus glucose, to a final concentration of 0.4 %; (3) verapamil at $\frac{1}{2}$ of MIC only; and (4) glucose to a final concentration of 0.4 % plus verapamil at $\frac{1}{2}$ of MIC. The final OD₆₀₀ of the bacterial suspension in the assay was 0.4. The fluorescence of ethidium bromide was measured, as described above. The fluorescence was acquired first, at the end of 15 seconds and at the end of every 30 seconds during the following 30 minutes. The efflux activity was quantified by comparing the fluorescence data obtained for the cells under conditions that allow maximum efflux (incubation at 37 °C in the presence of glucose and absence of a compound) against the data from the control tube that contains the ethidium bromide-load cells under conditions that inhibit the efflux (with a compound and absence of glucose).

4.3.12. Efflux pump gene expression.

(i) RNA extraction.

Total RNA was isolated from the cells using a GTC/Trizol based method. Briefly, cultures were centrifuged at 2940 x g during 16 minutes. Then, supernatant was removed leaving about 500 µl above the pellet. Five volumes of a 5 M GTC solution were added to each culture and incubated 5 minutes at room temperature. The mixture was then centrifuged at 2940 x g for 30 minutes and the supernatant was discarded. The pellet was resuspended in 1 ml of TRI reagent (Sigma). The suspension was transferred to lysis tubes containing glass beads (QIAGEN, GmbH, Hilden, Germany) and sonicated at 35 kHz (Gen-Probe, California, USA) during 3 x 5 minutes with 1 minute of cooling on

ice between intervals. The disrupted cells were incubated at room temperature during 10 minutes and centrifuged at 16060 x *g* for 45 seconds. Supernatant above the beads and cellular debris were transferred to a tube containing 300 µl of chloroform: isoamyl alcohol (24:1). Afterwards, the mixture was incubated during 7 minutes at room temperature followed by centrifugation at 16060 x *g* for 10 minutes. The top aqueous layer was then transferred to a clean tube containing an equal volume of isopropanol and the mixture incubated overnight at -20 °C. Precipitated nucleic acids were recovered by centrifugation at 18000 x *g* for 30 minutes at 4 °C and RNA pellet washed twice with 1 ml ice cold 70 % ethanol. RNA was dissolved in RNase-free water and contaminating chromosomal DNA was digested with RNase-free DNase I (QIAGEN) at room temperature during 30 minutes. RNA was purified with phenol: chloroform (4:1) during 10 minutes of incubation on ice and the mixture centrifuged at 18000 x *g* for 10 minutes at room temperature. The top aqueous layer was transferred to a new tube, to which 0.1 volumes of RNase-free sodium acetate pH 5.2 and 2.5 volumes of 100 % RNase-free ethanol were added. Samples were incubated overnight at -20°C. After this incubation, RNA was collected by centrifugation at 18000 x *g* for 30 minutes at 4 °C, and washed twice with 1 ml ice cold 70 % ethanol. The purified RNA was air-dried and then dissolved in 70 µl RNase-free water. Quantity and quality of the purified RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA).

(ii) *RT-qPCR analysis.*

The relative expression level of 10 efflux genes that code for membrane efflux transporters in *M. tuberculosis* and the transcriptional regulator *whiB7* (Table 6 and 7) were analysed by RT-qPCR in the strains exposed to sub-inhibitory concentrations of isoniazid or rifampicin. The primers employed are available upon request. The RT-qPCR procedure was performed in a Rotor-Gene™ 3000 thermocycler and followed the protocol recommended for use with the QuantiTect SYBR Green RT-PCR Kit (QIAGEN). The following amplification program was used: reverse transcription for 30 minutes at 50 °C; initial activation step for 15 min at 95°C; 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 30 seconds; a final extension step at 72 °C for 5 minutes; and an additional step

at 50 °C for 15 seconds followed by melt analysis (50-99 °C). The determination of the relative mRNA expression level was performed using the comparative quantification cycle (Cq) method (Livak and Schmittgen, 2001). The relative expression of the 11 genes was determined by comparison of the relative quantity of the respective mRNA in the presence of the antibiotic to the non-exposed condition. Each strain was assayed in triplicate using total RNA obtained from three independent cultures. Data was normalized to the *M. tuberculosis* 16S rDNA reference gene and presented as the mean fold change (\pm SD) compared with the control.

(iii) RNA extraction and RT-qPCR from infected macrophages.

Total RNA was isolated from control and isoniazid-treated *M. tuberculosis*-infected macrophages at day 1, 3, 5 and day 7 post-infection as described above. The RT-qPCR procedure was performed in a Rotor-GeneTM 3000 thermocycler and followed the protocol recommended for use with the QuantiTect SYBR Green RT-PCR Kit. The amplification program was as follows: reverse transcription for 30 minutes at 50 °C; initial activation step for 15 min at 95 °C; 45 cycles of denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute and extension at 72 °C for 1 minute; a final extension step at 72 °C for 5 minutes; and an additional step at 50 °C for 15 seconds followed by melt analysis. Data was normalized to the *M. tuberculosis* 16S rDNA reference gene and presented as the mean fold change (\pm SD) compared with the control.

4.3.13. Genetic characterization.

(i) Detection of mutations associated with resistance.

Genomic DNA was extracted using the QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions. The most common mutations in *katG* gene and *inhA* regulatory region were investigated using the Genotype MTBDR_{plus} (HainLifescience GmbH, Nehren, Germany). For the detection of the most frequent mutations in *gyrA* and *rrs* genes was used the Genotype MTBDR_s/ (HainLifescience) according to the manufacturer's instructions. Genomic analysis of the complete *inhA* gene and *eis* promoter region was performed by PCR amplification and DNA sequencing using the primers

described previously (Leung et al., 2006; Morlock et al., 2003; Perdigão et al., 2013). The reaction mixtures were prepared for a total reaction volume of 50 µl consisting of 1x Taq buffer (Fermentas, Ontario, Canada), 1.5 mM MgCl₂, 200 mM of each dNTP, 20 pmol of each primer (10 pmol for *eis*), 1.5U Taq DNA Polymerase (Fermentas), and 5 µl of chromosomal DNA. The PCR reactions were performed with the following amplification profile: initial denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 1 minute, annealing at 60 °C (56 °C for *eis*) for 1 minute and extension at 72 °C for 1 minute during 40 cycles. The final extension occurred at 72 °C for 10 minutes.

(ii) Detection of mutations in drug transporters.

The genes were analysed by PCR amplification and DNA sequencing to search for mutations. The primer sequences are available upon request from the authors. The reaction mixtures were prepared for a total reaction volume of 50 µl consisting of 1x Taq buffer (Fermentas), 1.5 mM MgCl₂, 200 mM of each dNTP, 20 pmol of each primer, 1.5U Taq DNA Polymerase (Fermentas), and 5 µl of chromosomal DNA. The PCR reactions were performed with the following amplification profile: initial denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 1 minute, primer dependent annealing temperature for 1 minute and extension at 72 °C for 1 minute during 40 cycles. The final extension occurred at 72 °C for 10 minutes.

(iii) Strain typing.

Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeats (MIRU-VNTR) genotyping was performed for each strain by multiplex PCR amplification 24 MIRU–VNTR loci, as described by Supply et al. (2006). The genotype of these strains was analysed using the MIRU-VNTR*plus* web application (Allix-Béguec et al., 2008) and the SITVITWEB database (Demay et al., 2012) when relevant.

4.4. Results

4.4.1. Ion channel blockers enhance the inhibitory activity of antibiotics against *M. tuberculosis*.

To determine whether the selected ion channel blockers known to act as efflux inhibitors show synergistic activity when combined with the antituberculosis drugs against the drug susceptible and resistant panel of MTB strains used in this study, the susceptibility of isoniazid, rifampicin, amikacin and ofloxacin was investigated in the presence and absence of subinhibitory concentrations of VP, TZ, CPZ, FPX and HAL. The *in vitro* interaction of two agents in combination can be interpreted as synergistic, indifferent, or antagonistic depending on whether the activity of the combination is superior than, comparable to, or smaller than, respectively, the activities of the individual drugs. The efficiency of the drug combination was determined through the calculation of the fractional inhibitory concentration (FIC) indexes (Lorian, 1996). The characterization of the nine MTB strains is presented in Table 4.1. The MICs and FICs obtained with the combinations of the inhibitors and the antibiotics against the MTB strains are listed in Table 4.2. As can be observed, the combination of isoniazid with the five compounds consistently demonstrated synergy, with FIC indexes ranging from 0.15 to 0.5, for the five clinical multi- and extensively drug resistant strains. For these, the MIC values of isoniazid were reduced from 2- to 20-fold depending on the strain and the inhibitor tested. No effect of the compounds was observed on the MIC of the isoniazid-monoresistant strains H37Rv Δ *katG* and 294/09 (single *inhA* promoter C-15T mutation). However, the combination between the phenothiazines with isoniazid demonstrated synergistic effect against the isoniazid-monoresistant strain 269/03 (single *katG* S315T mutation), being able to reduce 6-fold the MIC of isoniazid (FIC of 0.3). Contrary to the study by Warman et al. (2013) we did not detect antagonistic activity between isoniazid and the three phenothiazines in study. For rifampicin, significant synergistic activity was observed mainly with VP and CPZ. The combination of VP and rifampicin reduced the MIC values of rifampicin 16-fold in four strains and 4-fold in one strain, all carrying the *rpoB* S531L mutation. The FIC index for this combination was 0.06 and 0.25, respectively. CPZ reduced the MIC of rifampicin 16-fold in three strains and 4-fold in one strain, showing a FIC of 0.06

and 0.05, respectively. For strain 286/09, the MIC of rifampicin was reduced 20-fold by CPZ and FPX, and 4-fold by VP, TZ and HAL, originating a FIC of 0.05 and 0.25, respectively. For strains 149/09 and 29/12 rifampicin MIC values were reduced 8-fold. Only in one isolate (69/11), an heteroresistant strain for amikacin with simultaneous presence of drug susceptible and drug resistant genotype carrying the *rrs* A1401G mutation, was observed significant synergy when the combination of the compounds and amikacin was evaluated. For this strain, the MIC values were reduced 40-fold, to which corresponds a FIC index of 0.03 for all the five inhibitors tested. For the remaining three amikacin-resistant strains carrying the *rrs* A1401G mutation, none of the inhibitors, except CPZ, demonstrated any synergy. CPZ was found to have a FIC value of 0.13 for strains 149/09 and 29/12. The combination of the compounds with ofloxacin, for the three ofloxacin-resistant strains, was interpreted as being indifferent, with FIC indexes of 1 for all the compounds tested. Collectively, these results support the hypothesis that using synergistic combinations of inhibitors with antituberculosis drugs can significantly enhance the activities of first and second line antituberculosis drugs. However, can this enhancement bring back the clinical and therapeutic effect of the antibiotics that became non-effective due to the presence of mutations associated with resistance? To address this question, we performed quantitative drug susceptibility testing for the four antibiotics in the presence of the inhibitors to assess the levels resistance and correlate them with the mutations associated with clinical resistance.

Table 4.1. Molecular characterization, resistance pattern, and MIC values of the antibiotics and inhibitors for the nine *M. tuberculosis* strains.

Strain	Genotype	Resistance pattern	Gene mutations	Antibiotics (µg/ml)				Inhibitors (µg/ml)				
				INH	RIF	AMK	OFX	VP	TZ	CPZ	FPX	HAL
H37Rv	H37Rv	Susceptible	none	0.1	1	1	1	256	15	30	30	64
H37RvΔkatG	H37Rv	INH	<i>katG</i> deletion	128	1	1	1	128	15	30	30	32
82/09	LAM –Lisboa3	INH; RIF - MDR	<i>inhA</i> C-15T/S94A; <i>rpoB</i> S531L	3	320	1	1	256	15	30	30	64
149/09	LAM – Q1	INH; RIF; AMK; CAP; OFX - XDR	<i>inhA</i> C-15T/I194T; <i>rpoB</i> S531L; <i>gyrA</i> D94A; <i>rrs</i> A1401G	3	320	640	10	256	15	30	30	64
286/09	LAM –Lisboa3	INH; RIF; AMK; OFX – XDR	<i>inhA</i> C-15T/S94A; <i>rpoB</i> S531L; <i>gyrA</i> S91P; <i>eis</i> G-10A	20	80	4	10	128	15	30	30	32
69/11	Beijing	INH; RIF; AMK; CAP; OFX - XDR	<i>katG</i> S315T; <i>rpoB</i> S531L; <i>rrs</i> A1401G/wt*	20	320	40	1	256	15	30	30	32
29/12	LAM – Q1	INH; RIF; AMK; CAP; OFX - XDR	<i>inhA</i> C-15T/I194T; <i>rpoB</i> S531L; <i>gyrA</i> D94A; <i>rrs</i> A1401G	3	320	640	10	256	15	30	30	64
269/03	Unclustered*	INH	<i>katG</i> S315T	10	1	1	1	256	15	30	30	64
294/09	LAM – unclustered	INH	<i>inhA</i> C-15T	0.4	1	1	1	256	15	30	30	64

Δ, deletion; MDR, multidrug resistant; XDR, extensively drug resistant; INH, isoniazid; RIF, rifampicin; AMK, amikacin; CAP, capreomycin; OFX, ofloxacin; VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; FPX, flupenthixol; HAL, haloperidol. *mixed pattern: simultaneous presence of drug susceptible and drug resistant genotype. *VNTR international type 21 (see text for details). LAM, Latino-American-Mediterranean.

Table 4.2. Fractional inhibitory concentration (FIC) indexes of the inhibitors when combined with isoniazid, rifampicin, amikacin or ofloxacin, against the nine *M. tuberculosis* strains.

Drug combinations	<i>M. tuberculosis</i> strains [MIC in combination(µg/ml)/FIC]								
	H37Rv	H37Rv $\Delta katG$	82/09	149/09	286/09	69/11	29/12	269/03	294/09
INH	0.1/1	128/1	3/1	3/1	3/1	20/1	3/1	10/1	0.4/1
INH + VP	0.1/1	128/1	1/0.33	1/0.33	1/0.05	10/0.50	1/0.33	10/1	0.4/1
INH + TZ	0.1/1	128/1	1/0.33	1/0.33	20/1	3/0.15	3/1	3/0.30	0.4/1
INH + CPZ	0.1/1	128/1	1/0.33	1/0.33	1/0.05	3/0.15	1/0.33	3/0.30	0.4/1
INH + FPX	0.1/1	128/1	1/0.33	1/0.33	1/0.05	3/0.15	1/0.33	3/0.30	0.4/1
INH + HAL	0.1/1	128/1	1/0.33	1/0.33	1/0.05	10/0.50	1/0.33	10/1	0.4/1
RIF	1/1	-	320/1	320/1	80/1	320/1	320/1	-	-
RIF + VP	1/1	-	20/0.06	20/0.06	20/0.25	20/0.06	20/0.06	-	-
RIF + TZ	1/1	-	320/1	320/1	20/0.25	320/1	320/1	-	-
RIF + CPZ	1/1	-	320/1	20/0.06	4/0.05	20/0.06	20/0.06	-	-
RIF + FPX	1/1	-	320/1	320/1	4/0.05	320/1	320/1	-	-
RIF + HAL	1/1	-	320/1	320/1	20/0.25	320/1	320/1	-	-
AMK	1/1	-	-	640/1	4/1	40/1	640/1	-	-
AMK + VP	1/1	-	-	640/1	4/1	1/0.03	640/1	-	-
AMK+ TZ	1/1	-	-	640/1	4/1	1/0.03	640/1	-	-
AMK + CPZ	1/1	-	-	40/0.13	4/1	1/0.03	40/0.13	-	-
AMK + FPX	1/1	-	-	640/1	4/1	1/0.03	640/1	-	-
AMK + HAL	1/1	-	-	640/1	4/1	1/0.03	640/1	-	-
OFX	1/1	-	-	10/1	10/1	-	10/1	-	-
OFX + VP	1/1	-	-	10/1	10/1	-	10/1	-	-
OFX+ TZ	1/1	-	-	10/1	10/1	-	10/1	-	-
OFX + CPZ	1/1	-	-	10/1	10/1	-	10/1	-	-
OFX + FPX	1/1	-	-	10/1	10/1	-	10/1	-	-
OFX + HAL	1/1	-	-	10/1	10/1	-	10/1	-	-

Δ, deletion; INH, isoniazid; RIF, rifampicin; AMK, amikacin, OFX, ofloxacin; VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; FPX, flupenthixol; HAL, haloperidol. The lowest concentration tested corresponds to the critical concentration for each antibiotic (See Material and Methods section for details). The FIC was interpreted as: FIC≤0.5, synergism; >0.5-1, indifference; and >1, antagonism (Lorian, 1996).

4.4.2. Ion channel blockers reduce the resistance level of first and second line anti-tuberculosis drugs.

To assess the clinical effect of these compounds in combination with antituberculosis drugs in respect to their levels of resistance, and evaluate its correlation with the presence of mutations associated with resistance, the levels of resistance to isoniazid, rifampicin, amikacin, and ofloxacin were evaluated with the MGIT960 system and the TB eXIST software. The results are presented in Table 4.3. The addition of the compounds promotes a significant reduction of the resistance levels for all antibiotics except for ofloxacin.

In Figure 4.1 is shown the growth curves for strain 29/12, with and without VP, in the presence of isoniazid, as an example. This strain, which was initially resistant at 1 µg/ml of isoniazid, upon addition of ½ MIC of VP had his high level of resistance decreased to low level resistance (<1 µg/ml) (see full vs dotted pink line in Figure 4.1). The resistance levels to isoniazid were reduced from high level to low level in 4/8 of the resistant strains, with VP, FPX and CPZ and 2/8 with TZ. The strain is considered resistant when the number of drug-resistant bacteria present in the drug-containing tube at critical concentration is greater than 1 %, when compared with the drug-free growth control and correlates well with an effective clinical outcome (see Materials and Methods for critical concentrations) (Canetti et al., 1963). None of the strains had reversal of resistance since the resistance levels did not drop below the isoniazid critical concentration of 0.1 µg/ml (Table 4.3). Correlating the resistance levels to the antibiotics with the presence of mutations associated with resistance: four strains for which the resistance level was reduced in the presence of the inhibitors harbored double mutations in *inhA* gene, 2 with C-15T/S94A and 2 with C-15T/I194A replacements. The four remaining strains, where residual or no change of the resistance level was noticed in presence of the inhibitors, possessed single mutations: the C-15T mutation in *inhA* promoter in one strain, two strains with the S315T mutation in *katG*, and one with a complete deletion of *katG*. Strain H37Rv Δ *katG* was included in the study as a negative control, as it harbors a complete deletion of the *katG* gene and demonstrates no efflux activity (Machado et al., 2012). Regarding the strains with the *katG* S315T mutation, only a slight enhancement of the antimicrobial effect of isoniazid was

observed for both strains in the presence of the phenothiazines TZ, CPZ and FPX (Table 4.3). However, this potentiation was not enough to reach low level isoniazid resistance. Regarding the strain 294/09 with the C-15T mutation in the promoter region of *inhA* gene, low level resistance was due to the overexpression of *inhA* and the effect of this overexpression on isoniazid resistance cannot be overcome by the inhibitors tested. Concerning rifampicin, all strains presented high level resistance due to the presence of the mutation S531L in *rpoB*. The resistance level was remarkably reduced from high to low level in presence of VP (5/5), CPZ (4/5), and TZ, FPX and HAL (1/5) (Table 4.2 and Table 4.3). Four strains presented resistance to amikacin due to a mutation in a gene associated with amikacin resistance, three of them in *rrs1400* region and one strain in the *eis* promoter region. The high level resistance conferred by mutations in *rrs* could not be reduced in the presence of the inhibitors in 2/3 strains. Nevertheless, resistance to amikacin was reversed in strain 69/11. The amikacin resistance profile changed from high level resistance to susceptible in presence of the five compounds. In this case, *rrs* hybridization pattern given by Genotype MTBDRs/ assay indicated heteroresistance, *i.e.* the coexistence of a population of bacilli susceptible and resistant to amikacin. This is especially important since heteroresistance plays a role in the development of resistance in MTB (Hofmann-Thiel et al., 2009) and our data demonstrates that these inhibitors are capable to prevent the development of resistance to antituberculosis drugs. Amikacin resistance due to the mutation in *eis* promoter region could not be reduced. Again, this indicates that resistance due to the overexpression of the target gene cannot be altered. For ofloxacin, low level resistance was detected in three strains and correlated with mutations in *gyrA* gene. With this panel of strains, ofloxacin resistance could not be reduced by any of the inhibitors tested.

With this methodology, we have been able to demonstrate that the addition of ion channel blockers enhances the clinical effect of the main antituberculosis drugs, isoniazid and rifampicin, in multi- and extensively drug resistant strains, despite the presence of a mutation conferring resistance. However, their effect on isoniazid resistance seems to be limited to strains with mutations in *inhA* gene. The fact that strains changed their resistance profile from high level to low

level upon the addition of the inhibitors brings clinical implications. The serum concentrations of isoniazid and rifampicin attained *in vivo*, 7 and 10 µg/ml respectively, are much higher than the respective MICs for the majority of strains (Lorian, 1996). In the presence a high-level resistance strain (see Material and Methods section for breakpoints), these drugs are predicted to be useless. However, in the presence of a low-level resistance strain it is possible to reach high drug concentrations *in vivo*, since, the effectiveness of the synergistic combination of these inhibitors with conventional therapy, seen *in vitro*, is expected to be beneficial to reduce the resistance level of the strain.

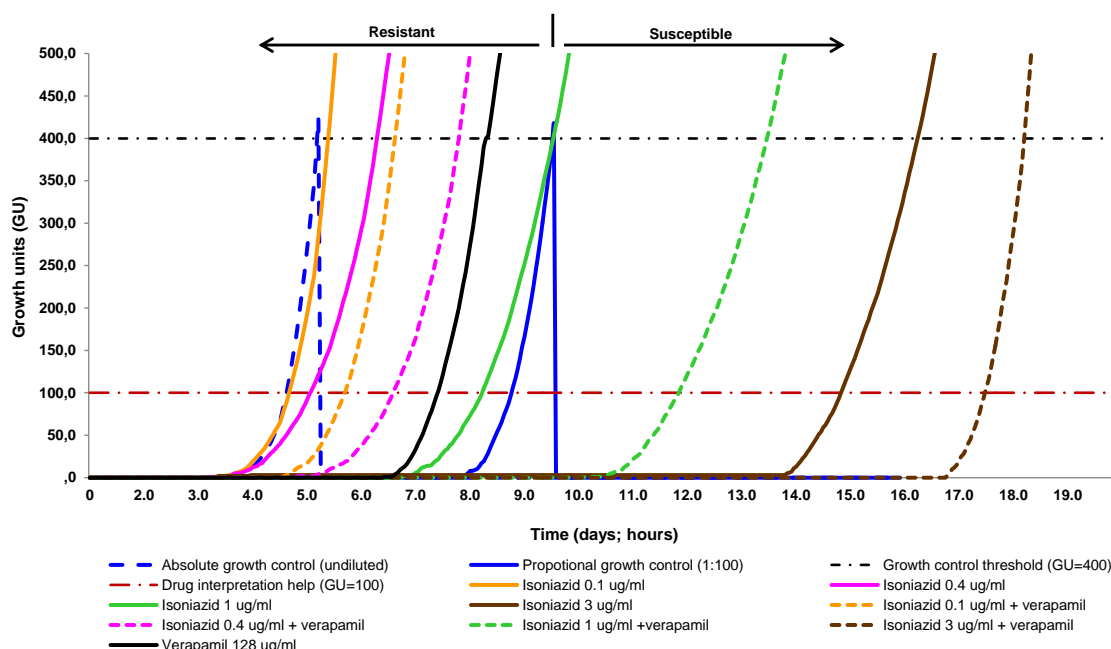


Figure 4.1. Quantitative drug susceptibility testing of isoniazid for the *M. tuberculosis* 29/12 strain, in the presence or absence of verapamil.

Quantitative drug susceptibility testing of isoniazid was conducted using the BACTEC 960 system and the Epicenter V5.80A software equipped with the TB eXIST module. Full and dotted dark-blue line indicates the drug-free proportional and absolute growth control, respectively. Coloured full lines correspond to the growth curves of the strain in the presence of isoniazid; the dotted lines correspond to the growth curves with isoniazid in combination with verapamil. At the time of growth of the proportional growth control (GU=400), the comparison between this tube and the tubes containing the drug(s) was performed. If the GU of the tubes containing the drug were >100, they were considered to be resistant to that concentration. If the GU of the tube containing the drug was <100 they were considered susceptible. Isoniazid was tested at 0.1, 0.4, 1, 3 and 10 µg/ml with and without ½ MIC verapamil.

Table 4.3. Molecular characterization, resistance pattern, and MIC values of the antibiotics and inhibitors for the nine *M. tuberculosis* strains.

Strain	Isoniazid							Rifampicin							Amikacin							Ofloxacin							
	Conc. (µg/ml)	-EI	+VP	+TZ	+CPZ	+FPX	+HAL	Conc. (µg/ml)	-EI	+V P	+TZ	+CP Z	+FP X	+HAL	Conc. (µg/ml)	-EI	+V P	+TZ	+CP Z	+FP X	+HAL	Conc. (µg/ml)	-EI	+V P	+TZ	+CP Z	+FP X	+HAL	
H37Rv	0.1	S	S	S	S	S	S	1	S	S	S	S	S	S	1	S	S	S	S	S	S	1	S	S	S	S	S	S	
	0.4	S	S	S	S	S	S	4	S	S	S	S	S	S	4	S	S	S	S	S	S	2	S	S	S	S	S	S	
	1	S	S	S	S	S	S	20	S	S	S	S	S	S	20	S	S	S	S	S	S	10	S	S	S	S	S	S	
	3	S	S	S	S	S	S																						
	10	S	S	S	S	S	S																						
H37Rv ΔkatG	0.1	R	R	R	R	R	R	ND							ND							ND							
	0.4	R	R	R	R	R	R																						
	1	R	R	R	R	R	R																						
	3	R	R	R	R	R	R																						
	10	R	R	R	R	R	R																						
82/09	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	ND							ND							
	0.4	R	R	R	R	R	R	4	R	R	R	R	R	R		4	R	R	R	R	R		R	2	R	R	R	R	R
	1	R	S	S	S	S	S	20	R	S	R	R	R	R		20	R	R	R	R	R		R	10	S	S	S	S	S
	3	S	S	S	S	S	S																						
	10	S	S	S	S	S	S																						
149/09	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R	
	0.4	R	R	R	R	R	R	4	R	R	R	R	R	R	4	R	R	R	R	R	R	2	R	R	R	R	R		
	1	R	S	S	S	S	S	20	R	S	R	S	R	R	20	R	R	R	R	R	R	10	S	S	S	S	S		
	3	S	S	S	S	S	S																						
	10	S	S	S	S	S	S																						
286/09	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R	
	0.4	R	R	R	R	R	R	4	R	R	R	S	S	R	4	S	S	S	S	S	S	2	R	R	R	R	R		
	1	R	S	R	R	S	S	20	R	S	S	S	S	S	20	S	S	S	S	S	S	10	S	R	S	S	S		
	3	R	S	R	R	S	S																						
	10	R	S	R	R	S	S																						
69/11	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	S	S	S	S	S	ND							
	0.4	R	R	R	R	R	R	4	R	R	R	R	R	4	R	S	S	S	S	S	S		2	R	R	R	R	R	
	1	R	R	R	R	R	R	20	R	S	R	S	R	20	R	S	S	S	S	S	S		10	R	S	S	S	S	
	3	R	R	S	S	S	S																						
	10	R	S	S	S	S	S																						
29/12	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R	
	0.4	R	R	R	R	R	R	4	R	R	R	R	R	4	R	R	R	R	R	R	2	R	R	R	R	R	R		
	1	R	S	R	R	S	S	20	R	S	R	S	R	20	R	R	R	R	R	R	10	S	S	S	S	S	S		
	3	S	S	S	S	S	S																						
	10	S	S	S	S	S	S																						
294/09	0.1	R	R	R	R	R	R	ND							ND							ND							
	0.4	S	S	S	S	S	S																						
	1	S	S	S	S	S	S																						
	3	S	S	S	S	S	S																						
	10	S	S	S	S	S	S																						
269/03	0.1	R	R	R	R	R	R	ND							ND							ND							
	0.4	R	R	R	R	R	R																						
	1	R	R	R	R	R	R																						
	3	R	R	S	S	S	S																						
	10	S	S	S	S	S	S																						

Δ, deletion; MDR, multidrug resistant; XDR, extensively drug resistant; INH, isoniazid; RIF, rifampicin; AMK, amikacin; CAP, capreomycin; OFX, ofloxacin; VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; FPX, flupentixol; HAL, haloperidol. *mixed pattern: simultaneous presence of drug susceptible and drug resistant genotype. *VNTR international type 21 (see text for details). LAM, Latino-American-Mediterranean.

4.4.3. The ion channel blockers display rapid and high killing activity.

One of the prerequisites for the development of new anti-tuberculosis drugs includes the need for compounds that have bactericidal activity in order to prevent the development of drug resistance and act rapidly in order to reduce the duration of the treatment (Ginsberg, 2010). To characterize the bactericidal activity of the ion channel blockers and evaluate if they can enhance the bactericidal efficacy of the anti-tuberculosis drugs, we measured their anti-mycobacterial killing activity against the strains H37Rv (drug-susceptible) and 149/09 (drug-resistant) through time-kill studies. The time-kill kinetics of the compounds and antibiotics on MTB cultures are shown in Figure 4.2. All the compounds exhibit time-dependent bactericidal activity. After seven days, FPX, CPZ, TZ, HAL, and VP were able to eliminate all MTB cells. FPX reduced the cell number to zero at day 1, CPZ and TZ at day 2, and VP and HAL at day 7. Moreover, at the end of 100 days all cultures remain negative. Isoniazid, rifampicin, amikacin, and ofloxacin were not able to reduce the viable number of MTB cells to zero by the end of the seven days. On the seventh day of incubation, a $3\log_{10}$ CFU/ml decrease by rifampicin, amikacin, and ofloxacin was observed. For isoniazid was notice a $2\log_{10}$ reduction in the same time frame.

The most effective ion channel blocker previously tested, VP, was selected for the evaluation of the synergistic effect with the antibiotics through time-kill studies. The data presented in Figure 4.2B corresponds to the measurement of time-kill kinetics for VP with isoniazid, rifampicin, amikacin and ofloxacin for H37Rv. It can be observed that the activity of the antibiotics was strongly potentiated. By the end of day seven no bacteria remained viable for the five combinations tested. The combination of VP plus amikacin reduced the CFU/ml to zero at day two, VP and rifampicin at day three, VP and ofloxacin also at day three and VP and isoniazid at day seven. Similar results were obtained for the multidrug resistant strain (data not shown). These results demonstrate that FPX, CPZ, TZ, HAL, and VP have direct bactericidal activity and when combined with the antibiotics they can stimulate a rapid and high killing effect against both drug susceptible and multidrug resistant MTB, therefore fulfilling both criteria mentioned above.

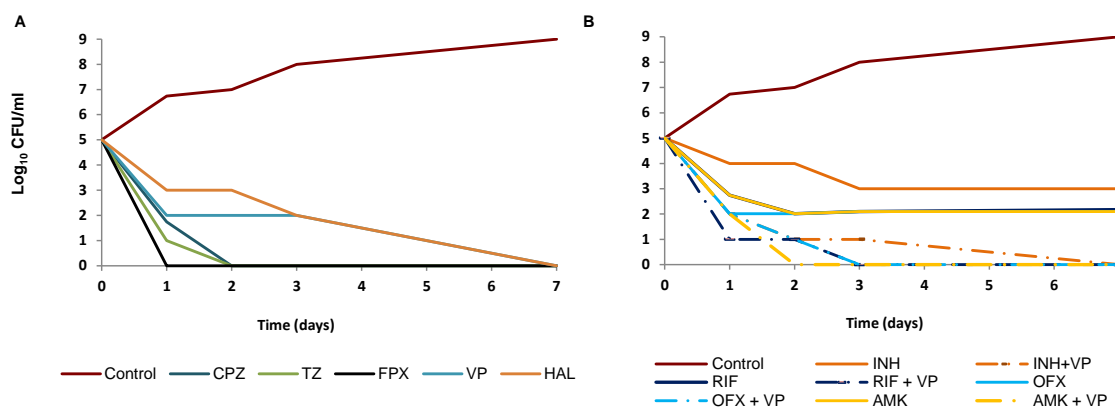


Figure 4.2. Time-kill curves for flupenthixol, chlorpromazine, thioridazine, haloperidol, verapamil and the anti-tuberculosis drugs isoniazid, rifampicin, amikacin and ofloxacin. Compounds were used at 5X MIC and CFU/ml was determined at day 1, 2, 3 and 7. A) Killing activity of efflux inhibitors; B) Killing activity of the antibiotics in the presence or absence of verapamil. In the figure, is represented the assay for the H37Rv strain. Data corresponds to the average of two experiments. VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; HAL, haloperidol; FPX, flupenthixol; INH, isoniazid; RIF, rifampicin; AMK, amikacin; OFX, ofloxacin.

4.4.4. ATP depletion occurs in response to the treatment with the ion channel blockers.

To determine whether the treatment with the ion channel blockers could have an effect on intracellular ATP levels, we monitored the intracellular ATP levels over seven days. ATP levels and killing activity was recorded after day 1, 2, 3 and 7 of exposure to the compounds. The mycobacterial ATP levels in cultures of *M. tuberculosis* H37Rv, exposed to 5X the MIC for each compound, are shown in Fig. 3A and viability, in Figure 4.3B. A rapid drop in intracellular ATP levels of *M. tuberculosis* H37Rv was noticed after 1 day of exposure with all compounds, which progressively falls until the seventh day of exposure. This decrease in the ATP levels was correlated with an increased killing activity. Isoniazid and rifampicin do not have a significant effect on the ATP levels. These results confirm that ATP depletion occurs in consequence of exposure to the compounds and indicates that the metabolic state of the cells is directly affected by them with anticipated effects on the available bacterial energy for active efflux.

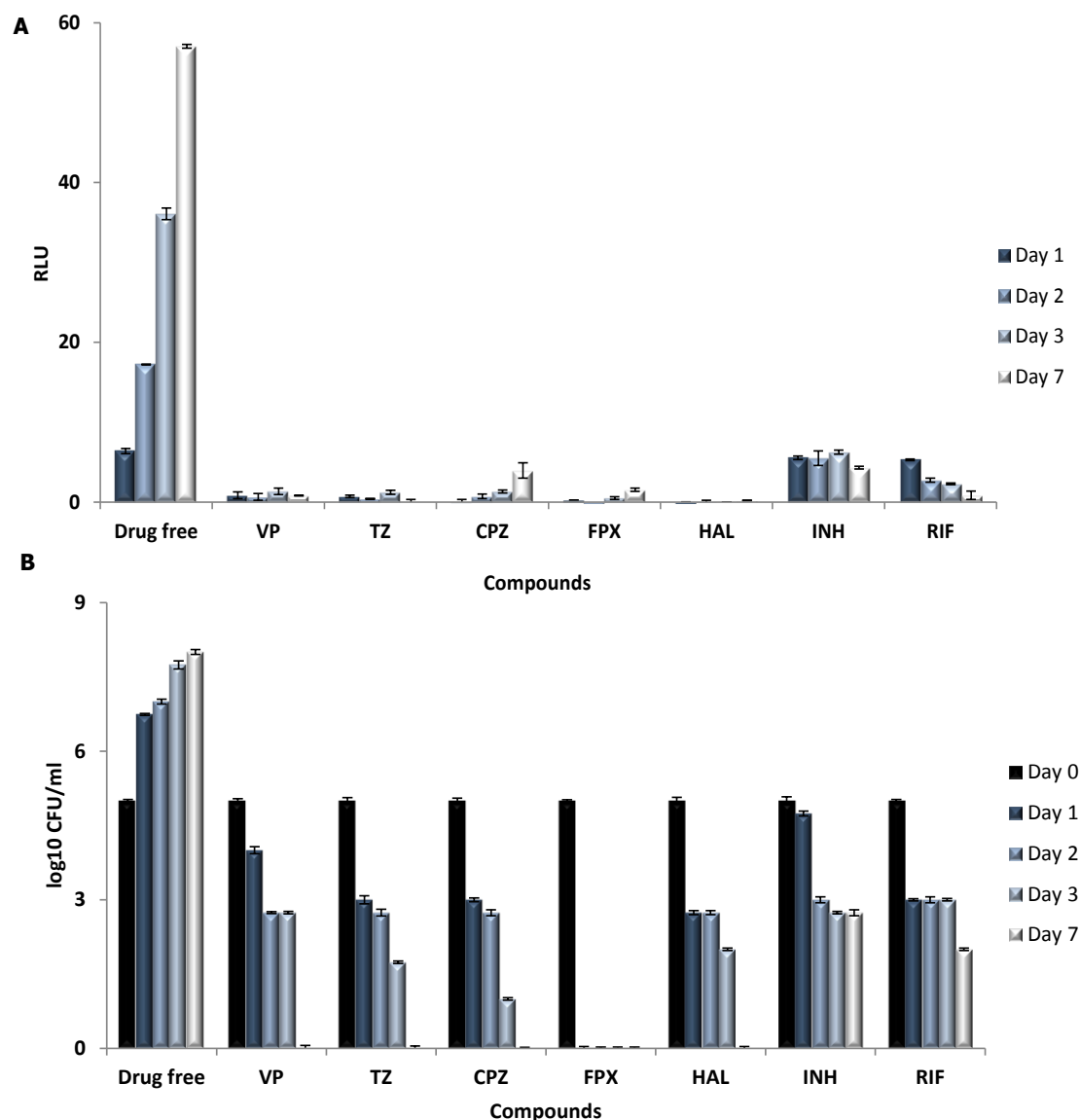


Figure 4.3. Mycobacterial intracellular ATP levels and viability. Cultures of *M. tuberculosis* H37Rv were exposed to the inhibitors during 7 days. ATP was determined by using a luciferin-luciferase bioluminescence detection system at day 1, 2, 3 and 7. Cell viability was measured in parallel with MGIT960 system as described in Material and Methods. Isoniazid (INH) and rifampicin (RIF) were used as controls.

4.4.5. Inhibition of ethidium bromide efflux demonstrates the role of the ion channel blockers as efflux inhibitors.

The ability of VP, TZ, CPZ, FPX and HAL to inhibit the efflux activity of the MTB strains used in this study was carried out using an semi-automated ethidium bromide fluorometric method (Viveiros et al., 2010) with a modified protocol for MTB (Machado et al., 2012). When data for the clinical strains were compared with those for the H37Rv strain, different levels of accumulation were observed

for all isolates. The results showed that the clinical strains are able to handle higher ethidium bromide concentrations than H37Rv. This means that the clinical strains have higher efflux capacity than that of the reference strain as previously described (Machado et al., 2012). These assays were then performed in the presence of the same compounds at sub-inhibitory concentrations. The inhibitory indexes of activity of each compound against the MTB strains are presented in Table 4.4. The relative final fluorescence index corresponds to a measure of how efficient is the inhibitory effect of the compounds on efflux by comparison of the final fluorescence of the cells exposed to ethidium bromide plus the compound against cells exposed only to ethidium bromide (taken as 0). The accumulation of ethidium bromide increased in presence of all inhibitors. VP promoted the largest increase in accumulation in all strains, except H37Rv Δ katG (7/8), followed by the phenothiazines. Among this class of compounds, FPX demonstrated the highest inhibitory effect (3/7), followed by TZ and CPZ, which demonstrated similar activity against 2/7 of the multidrug resistant strains. HAL was the compound that demonstrated the lowest effect on the accumulation of ethidium bromide. The multidrug resistant strains showed significantly higher levels of ethidium bromide accumulation in presence of the inhibitors, whereas the clinical isoniazid monoresistant strains accumulated significantly lower levels of ethidium bromide when compared with those from the multidrug resistant strains. For the *katG* deleted strain, only a minor effect was observed with VP.

To directly assess efflux, we selected VP to measure the inhibition of ethidium bromide efflux on these strains. The results showed that the ethidium bromide efflux is greatly inhibited in the presence of VP. Therefore, from the analysis of Figure 4.4B we correlate these results with the effect on the ethidium bromide accumulation observed in Figure 4.4A and conclude that the inhibition of efflux pumps retain ethidium bromide inside the cells. All together, the effects we observed on the MICs followed the same pattern as in the ethidium bromide accumulation promoted by the inhibitors, correlating the resistance levels of these strains with their ability to efflux noxious compounds.

Table 4.4. Relative final fluorescence (RFF) based on the accumulation of ethidium bromide for the *M. tuberculosis* strains in the presence of the inhibitors.

Strain	RFF of the inhibitors				
	VP	TZ	CPZ	FPX	HAL
H37Rv	1.58	0.97	0.84	0.90	0.32
H37Rv Δ katG	0.37	0.09	0.03	0.15	-0.06
82/09	2.17	0.96	0.63	1.24	0.44
149/09	2.31	1.13	1.16	1.45	0.58
69/11	1.19	0.23	0.47	0.95	0.21
29/12	2.00	1.14	1.04	1.03	0.24
294/09	1.21	0.52	0.44	0.98	-0.18
269/03	0.73	0.17	0.07	0.35	-0.02

VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; FPX, flupenthixol; HAL, haloperidol. Strain 286/09 was not evaluated due to poor growth under the conditions required for the accumulation assays. Results in bold face indicate significant inhibition.

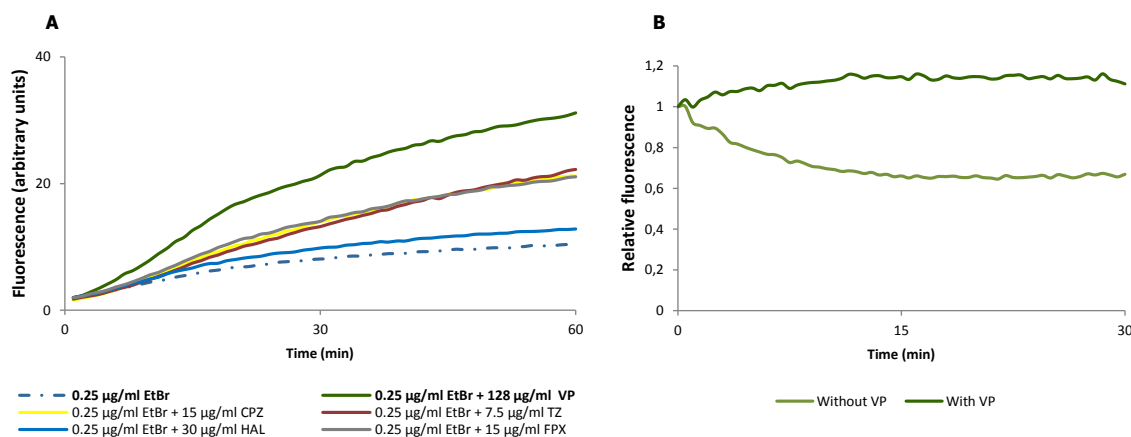


Figure 4.4. Effect of the inhibitors on the accumulation and efflux of ethidium bromide by the *M. tuberculosis* strains.

In the figure is presented an assay for the strain 29/12 as an example. A) Accumulation and B) efflux of ethidium bromide. Ethidium bromide was tested at 0.25 µg/ml. Efflux inhibitors were tested at ½ the MIC. EtBr, ethidium bromide; VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; HAL, haloperidol; FPX, flupenthixol.

4.4.6. Overexpression of efflux pumps in response to the antibiotic pressure leads to increased tolerance towards antibiotics.

In a previous work we have been able to correlate the increased ethidium bromide efflux in MTB with efflux-mediated increased isoniazid resistance as the result of the activity of several overexpressed efflux pumps working in combination (Machado et al., 2012). Here, we assess the contribution of the MTB membrane transporters for the increased levels of resistance towards isoniazid and rifampicin previously seen for the multidrug resistant MTB strains. For that, these strains were exposed to sub-inhibitory concentrations of isoniazid or rifampicin and the expression levels of genes that code for 10 efflux pumps and the global regulator *whiB7* were evaluated by RT-qPCR. Table 4.5 and Table 4.6 show the results obtained for the quantification of gene expression of the cells exposed to isoniazid and rifampicin, respectively. As can be observed, the majority of the efflux genes are overexpressed in response to isoniazid and rifampicin exposure. Although we cannot observe a specific pattern of expression, the genes *mmr*, *mmp17*, *p55*, *Rv1217c* and *efpA* showed a significantly increased in their expression levels in the presence of isoniazid. Regarding the exposure to rifampicin, we can observe an increase in the expression of the majority of the genes albeit at lower absolute levels than those obtained for the isoniazid exposed strains. Of all pumps studied, P55 a major facilitator superfamily efflux-pump described in 2001 by Silva et al. (2001), was found to be consistently expressed in all strains, presenting a 1.54 to 9.88-fold increase in presence of rifampicin.

Table 4.5. Average quantification of the relative expression level, by RT-qPCR, of the genes that code for efflux pumps in the *M. tuberculosis* exposed to isoniazid.

Gene	Relative expression level \pm SD						
	82/09	149/09	286/09	29/12	69/11	294/09	269/03
<i>mmr</i>	6.29 \pm 3.23	2.22 \pm 0.11	0.78 \pm 0.22	1.63 \pm 0.16	0.76 \pm 0.07	0.64 \pm 0.03	0.74 \pm 0.04
<i>mmpl7</i>	0.49 \pm 0.38	2.49 \pm 0.49	2.23 \pm 0.33	4.03 \pm 2.87	0.76 \pm 0.00	0.83 \pm 0.24	0.72 \pm 0.21
<i>Rv1258c</i>	0.79 \pm 0.40	1.34 \pm 0.26	0.48 \pm 0.40	2.22 \pm 0.11	0.79 \pm 0.30	0.85 \pm 0.12	0.64 \pm 0.03
<i>p55</i>	26.06 \pm 2.55	1.65 \pm 0.92	0.67 \pm 0.13	4.04 \pm 0.78	0.86 \pm 0.41	1.38 \pm 0.20	0.69 \pm 0.17
<i>whiB7</i>	3.38 \pm 1.29	0.19 \pm 0.01	0.71 \pm 0.07	0.87 \pm 0.00	0.61 \pm 0.15	0.67 \pm 0.13	2.55 \pm 1.32
<i>1217c</i>	5.94 \pm 1.44	0.08 \pm 0.01	0.60 \pm 0.30	5.12 \pm 1.96	0.69 \pm 0.17	1.33 \pm 0.26	0.62 \pm 0.06
<i>1218c</i>	1.36 \pm 0.91	0.04 \pm 0.00	0.50 \pm 0.00	2.17 \pm 0.42	0.44 \pm 0.08	1.28 \pm 0.06	0.72 \pm 0.13
<i>efpA</i>	10.11 \pm 3.82	1.38 \pm 0.21	0.80 \pm 0.18	1.76 \pm 0.34	1.39 \pm 0.33	0.81 \pm 0.00	1.52 \pm 0.15
<i>Rv2459</i>	0.44 \pm 0.15	1.62 \pm 0.00	0.44 \pm 0.26	1.86 \pm 0.63	0.68 \pm 0.19	0.73 \pm 0.04	0.94 \pm 0.09
<i>pstB</i>	1.54 \pm 0.66	1.12 \pm 0.16	0.49 \pm 0.24	1.94 \pm 0.28	0.74 \pm 0.11	1.08 \pm 0.21	0.55 \pm 0.11
<i>iniA</i>	0.26 \pm 0.05	0.05 \pm 0.00	1.41 \pm 0.23	1.53 \pm 0.30	0.46 \pm 0.02	1.00 \pm 0.00	0.38 \pm 0.18

The relative expression of the 11 genes was determined by comparison of the relative quantity of the respective mRNA in the presence of isoniazid to the non-exposed condition. Data was normalized to the *M. tuberculosis* 16S rDNA reference gene and presented as the mean fold change (\pm SD) compared with the control. Results in bold were considered overexpressed.

Table 4.6. Average quantification of the relative expression level, by RT-qPCR, of the genes that code for efflux pumps in the *M. tuberculosis* exposed to rifampicin.

Gene	Relative expression level \pm SD				
	82/09	149/09	286/09	69/11	29/12
<i>mmr</i>	1.31 \pm 0.62	0.73 \pm 0.49	2.49 \pm 0.49	1.00 \pm 0.33	0.71 \pm 0.00
<i>mmpl7</i>	1.85 \pm 0.87	0.46 \pm 0.23	3.23 \pm 1.09	0.39 \pm 0.08	1.02 \pm 0.30
<i>Rv1258c</i>	1.40 \pm 0.66	1.31 \pm 0.44	0.94 \pm 0.18	0.20 \pm 0.03	0.93 \pm 0.31
<i>p55</i>	4.27 \pm 1.44	2.30 \pm 0.23	9.88 \pm 0.97	1.54 \pm 0.66	2.59 \pm 0.63
<i>whiB7</i>	0.87 \pm 0.29	1.61 \pm 0.76	1.04 \pm 0.16	2.47 \pm 0.24	1.83 \pm 0.44
<i>1217c</i>	0.71 \pm 0.07	0.52 \pm 0.07	0.98 \pm 0.76	0.14 \pm 0.08	1.12 \pm 0.57
<i>1218c</i>	0.72 \pm 0.40	0.36 \pm 0.12	0.69 \pm 0.10	0.51 \pm 0.05	1.44 \pm 0.62
<i>efpA</i>	1.94 \pm 0.09	1.83 \pm 0.44	1.41 \pm 0.47	1.81 \pm 0.27	3.47 \pm 1.17
<i>Rv2459</i>	0.69 \pm 0.35	0.41 \pm 0.04	0.74 \pm 0.04	0.28 \pm 0.08	1.08 \pm 0.21
<i>pstB</i>	0.41 \pm 0.30	0.46 \pm 0.11	0.47 \pm 0.04	0.22 \pm 0.01	1.41 \pm 0.47
<i>iniA</i>	1.94 \pm 0.28	0.89 \pm 0.25	54.50 \pm 21.35	1.47 \pm 0.21	3.11 \pm 1.20

The relative expression of the 11 genes was determined by comparison of the relative quantity of the respective mRNA in the presence of rifampicin to the non-exposed condition. Data was normalized to the *M. tuberculosis* 16S rDNA reference gene and presented as the mean fold change (\pm SD) compared with the control. Results in bold were considered overexpressed.

This systematic increase in efflux pumps gene expression after isoniazid and rifampicin exposure, correlated with increased efflux and antibiotic resistance, can occur due two possible mechanisms. First, increase in gene expression due to a mutation in a regulatory or promoter region of the efflux-pump gene leading to constitutive gene expression and second, this increase can be due to the induction promoted by the compounds. To clarify which inductive mechanism is promoting this overexpression, we searched for mutations on the transporters studied. Despite missense and silent mutations in the coding regions of these genes, no other mutations were found on putative promoter or regulatory regions in these strains (Table 4.7). This indicates that the overexpression detected in these strains is induced by the antibiotic substrates and is not constitutive.

Table 4.7. Genetic characterization of the efflux transporters studied.

Gene	Strain						
	82/09	149/09	286/09	69/11	29/12	269/03	294/09
<i>iniA</i>	del GCG after nt 285	wt	del GCG after nt 285	wt	wt	wt	wt
<i>Rv1258c</i>	wt	wt	wt	C194del	wt	wt	wt
<i>p55</i>	wt	Q506	Q506	Q506	Q506	wt	Q506
<i>Rv1217c</i>	wt	A531	A531	A173T/S204/A531	A531	wt	wt
<i>Rv1218c</i>	wt	wt	wt	Q243R	wt	wt	wt
<i>pstB</i>	T61M	T61M	T61M	wt	T61M	wt	T61M

Wt, wild type.

Afterwards, we sought to see if the expression of some of these pumps can be induced upon macrophage residence. As proof of evidence, we selected one multidrug resistant strain, the MTB 82/09, to study the expression of five efflux genes, *mmr*, *mmpI7*, *Rv1258c*, *p55* and *efpA*, and determined their expression level upon exposure to isoniazid inside macrophages. The results show that MTB efflux pumps are also expressed inside the macrophages upon exposure to isoniazid (Figure 4.5) demonstrating that MTB drug resistance in macrophages is mediated by the expression of pumps that help the bacteria to

reduce the intracellular concentration of the antibiotic. Our results are in agreement with other previously reported data (Adams et al., 2011, 2014). Collectively, these results reinforce the concept that induction of efflux pumps is one of the mechanisms involved in drug resistance in MTB. Similar to our previous work, here we see that efflux pumps are promiscuous in their activity as we could not associate extrusion of isoniazid or rifampicin to a specific pump (Machado et al., 2012). Moreover, the results indicate that the compounds are not acting on a specific pump but on multiple pumps, and induction of efflux pumps seems to be a general stress response to the presence of noxious compounds, instead of a specific response of a specific pump. The differences observed in the gene expression profiles in each clinical strain most probably reflect their history of different antibiotic pressures in the clinical settings.

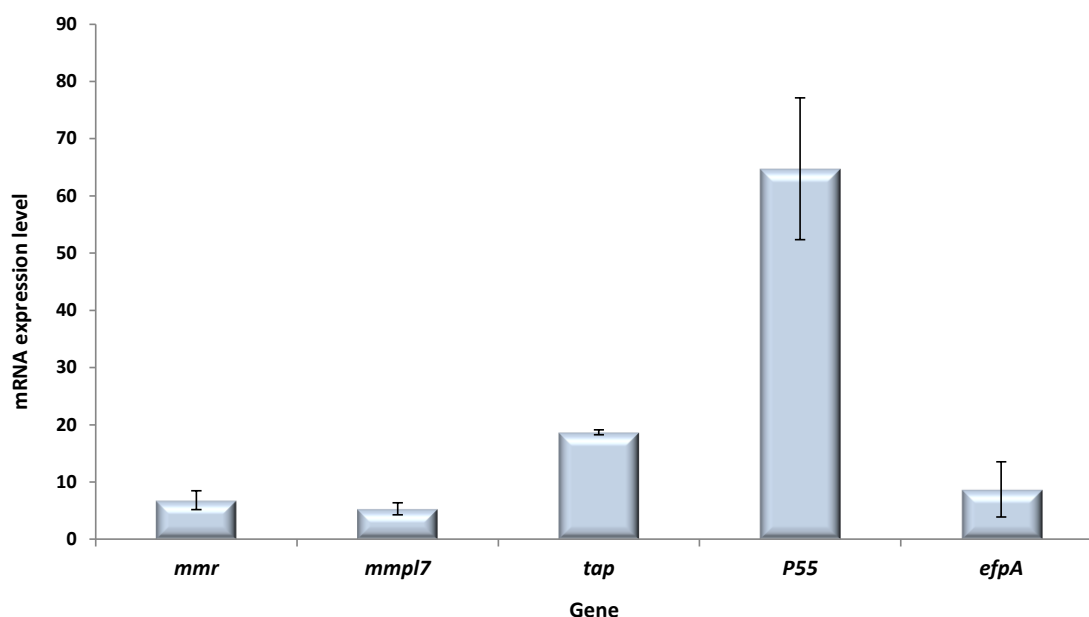


Figure 4.5. Relative expression of genes coding for efflux pumps in *M. tuberculosis* 82/09 strain during intracellular growth when exposed to isoniazid. *M. tuberculosis* infected macrophages were exposed to 0.1 μ g/ml isoniazid during 3 days. Relative expression of isoniazid-treated cells was compared with untreated control cells.

4.4.7. The ion channel blockers possess antibacterial activity against intracellular *M. tuberculosis*.

Macrophages are phagocytic cells devoted to kill microorganisms. MTB has evolved a large and diverse array of virulence factors well suited to interfere with or stimulate a variety of host-cell responses in order to invade, survive and replicate within macrophages. Therefore compounds to be applied as antimicrobials or enhancers of antimycobacterial agents should demonstrate activity against intracellular MTB. For this purpose, we examined the intracellular activity of VP, FPX, TZ, CPZ, and HAL, and their ability to potentiate isoniazid activity, on human monocyte-derived macrophages. Isoniazid was chosen due to be one of the most effective anti-tuberculosis agents known to date (Mitchison and Davies, 2012). We used one susceptible strain, H37Rv, and four isoniazid resistant strains with commonly described isoniazid-associated resistance mutations in *katG* and *inhA*. The intracellular killing activity promoted by the compounds and their combined effect with isoniazid after 3 days of infection is presented in Figure 4.6. We found that all the compounds impaired growth of intracellular MTB for both wild-type and resistant strains while had the highest activity. TZ enhanced the killing of mycobacteria by 58 % to 88 %, whereas the remaining compounds demonstrated rates of killing of 12 % to 64 % with VP, 37 % to 60 % with CPZ, 27 % to 43 % with FPX, and 11 % to 37 % with HAL. The co-administration of isoniazid with any of the compounds studied in this work conducted to a strong enhancement of the killing activity against the H37Rv strain. Conversely, no additional killing was observed in the isoniazid-resistant strains when isoniazid was used in combination with the compounds, as expected. This result is in agreement with the fact that all strains, except H37Rv, harbored a mutation that confers isoniazid resistance. Collectively, these results allow us to show that VP, FPX, TZ, CPZ, and HAL strengthen the killing activity against intracellular MTB.

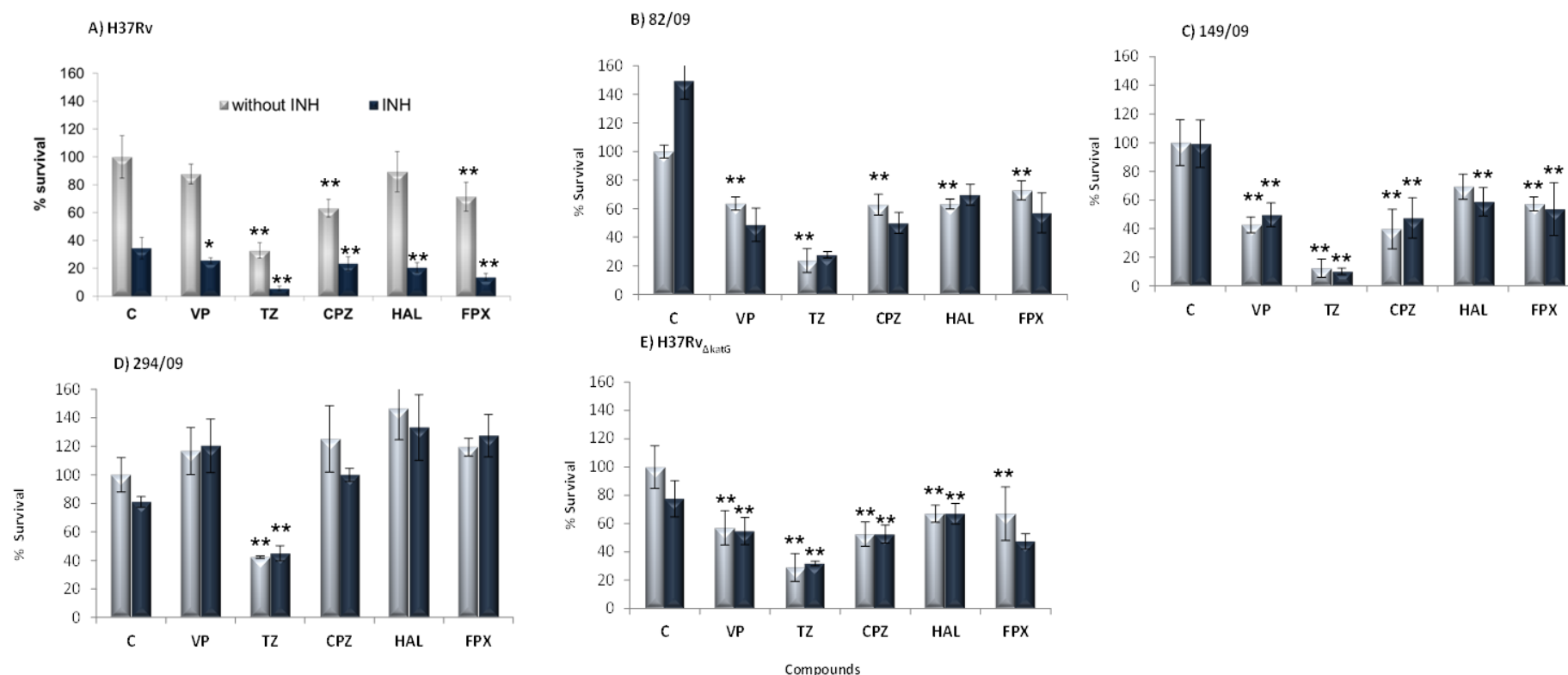


Figure 4.6. Anti-mycobacterial activity of the ion channel blockers on *M. tuberculosis* infected macrophages.

Effect of the inhibitors on the intracellular survival of *M. tuberculosis* within human monocyte-derived macrophages 3 days post infection. INH was tested at 0.1 $\mu\text{g/ml}$; VP, 10 $\mu\text{g/ml}$; TZ, 2.5 $\mu\text{g/ml}$; CPZ, 1.25 $\mu\text{g/ml}$; HAL, 1.25 $\mu\text{g/ml}$; FPX, 1.25 $\mu\text{g/ml}$. INH: isoniazid; VP: verapamil; TZ: thioridazine; CPZ: chlorpromazine; HAL: haloperidol; FPX: flupenthixol. The results are presented as mean percentage of the survival \pm SD (* $p < 0.05$; ** $p < 0.01$)

4.4.8. The ion channel blockers promote intracellular acidification.

One of the virulence mechanisms of MTB in macrophages is the blockade of MTB-containing phagosome maturation into a phagolysosome. Consequently, the bacilli will persist within a vacuole that does not acidify neither acquires proteolytic enzymes, although even if acquired they would not be active due to lack acidification. It was previously demonstrated that restriction of mycobacterial growth requires acidification of phagosomes (Jordao et al., 2008; Welin et al., 2011). Since we showed the inhibitors to possess efflux pumps inhibitor activity in bacteria, we hypothesized these compounds may have a similar ion channel blocker effect directly on the host vacuolar membrane inducing phagosome acidification and consequently enhancing macrophage-mediated killing of mycobacteria. To test this hypothesis, human monocyte-derived macrophages infected with *M. bovis* BCG (BCG) expressing GFP were treated with the compounds and stained with LysoTracker Red, a lysosomotropic probe that emits fluorescence at low pH. We used BCG because similarly to MTB, BCG blocks phagosome acidification (Jordao et al., 2008) while the confocal or flow cytometry analysis of live cells needn't be performed in a BSL-3 environment. In addition BCG is more sensitive to the killing effects of low pH than MTB (Jordao et al., 2008) being a better model to sense the killing effects of phagosome acidification. As shown in Figure 4.7, comparing control infected cells with ion channel blocker-treated cells, the LysoTracker staining revealed an increased co-localization of BCG with acidic vesicles. In order to determine if the acidification of BCG-containing phagosomes is occurring due to increased phagosome maturation we also stained BCG infected macrophages with LAMP-2, a characteristic marker of late phagosomes yet we could not observe co-localization of BCG with LAMP-2 (data not shown) revealing that the ion channel blockers are not increasing phagosome maturation and thus, the acidification observed is occurring via an independent mechanism.

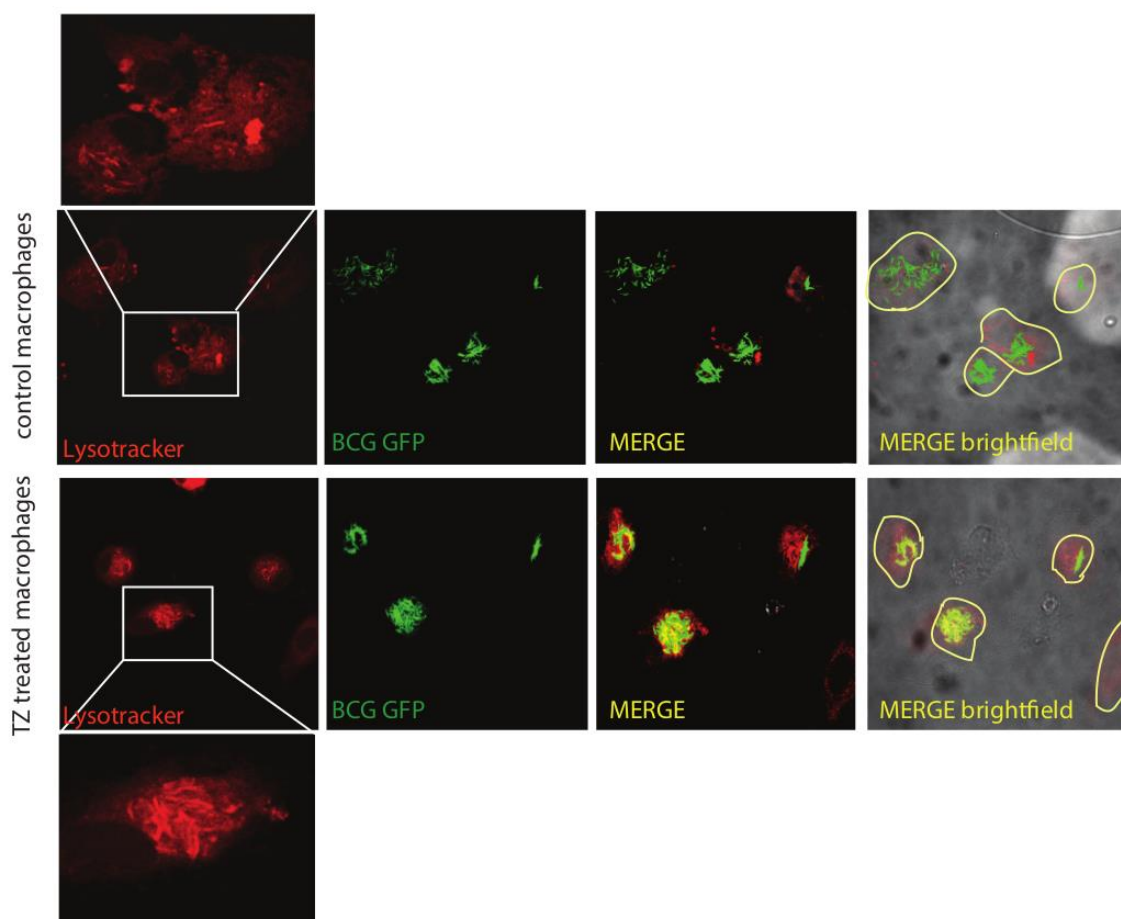


Figure 4.7. Phagosomal acidification in *BCG* GFP-infected macrophages stained with LysoTracker. Human macrophages were infected with *BCG*-GFP and treated thioridazine (TZ) 2.5 μ g/ml. Cells were stained with LysoTracker Red and analyzed by fluorescence microscopy.

To further quantify the extent of vesicle acidification in human monocyte-derived macrophages we measured the accumulation of LysoTracker Red by flow cytometry and LysoSensor Green by fluorescence microscopy. Unlike LysoTracker, LysoSensor correlates an increase in fluorescence with a decrease in pH. Using this combined strategy we could use flow cytometry to quantify the increase in acidic vesicles inside macrophages while using fluorescence microscopy to assess if vesicular pH as decreasing. First we analysed the effects of the compounds in non-infected cells. The results showed that all the compounds significantly increased the percentage of cells stained positive for LysoTracker RED (Figure 4.8A, dot plots). Next, we measured the increase on the overall number of LysoTracker RED stained vesicles per cell (average fluorescence intensity), and we observed that all the

compounds increase the number of stained vesicles (Figure 4.8A, bar plot). In both cases, VP, TZ and HAL induced the strongest effects. These results clearly show that the compounds are able to induce the increase of acidified vesicles in macrophages. Then, we analysed the fluorescence intensity of Lysosensor in treated cells (Figure 4.8B). As is observable, treated macrophages display a severe increase in fluorescent vesicles. This suggests that the ion channel blockers are inducing a general increase of vesicular acidification in the macrophages. Has previously published by our group (Jordao et al., 2008), pH alone may have a killing effect on mycobacteria-containing phagosomes. While BCG is more sensitive to acidification, MTB is able to survive, to some extent, in the acidic environment of the phagolysosome of activated macrophages and its survival or elimination depends on the ability of the macrophage to activate its pH-dependent lysosomal hydrolases (Vandal et al., 2008). Following this rationale, we assessed the impact of pH on the survival of MTB *in vitro* and we observed that MTB is able to persist in an acidic environment and that acidification only causes a significant reduction in MTB survival in non-physiological pH values (Figure 4.9).

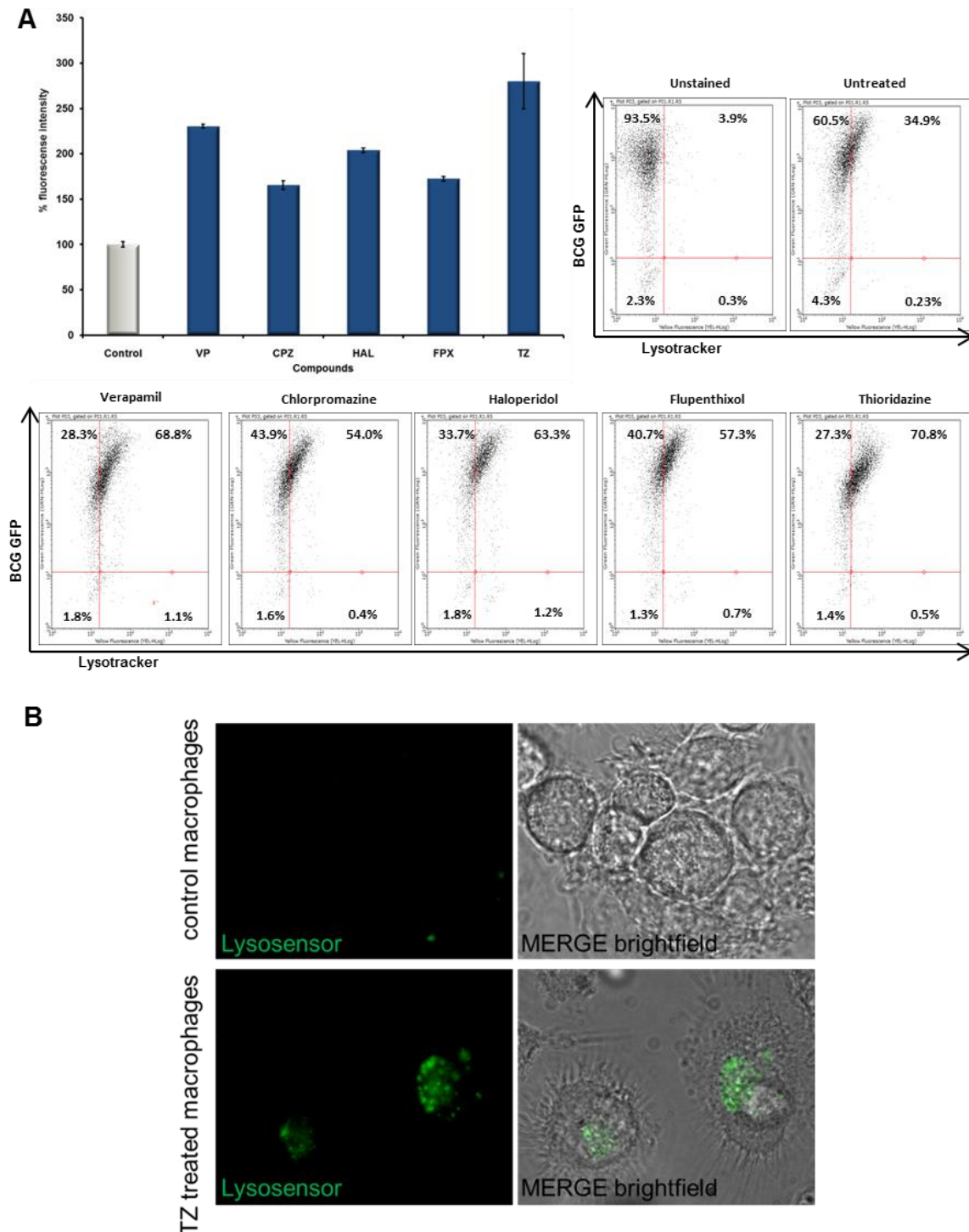


Figure 4.8. Relative quantification of phagosomal acidification in *BCG* GFP-infected macrophages stained with LysoTracker or Lysosensor.

Human macrophages were infected with *BCG*-GFP and treated with verapamil (VP) 10 µg/ml, thioridazine (TZ) 2.5 µg/ml, chlorpromazine (CPZ) 1.25 µg/ml, flupenthixol (FPX) 1.25 µg/ml, and haloperidol (HAL) 1.25 µg/ml, stained with LysoTracker Red and analyzed by flow cytometry (A) or with Lysosensor Green and analyzed by fluorescence microscopy (B). All treatments were statistically significant ($p < 0.001$).

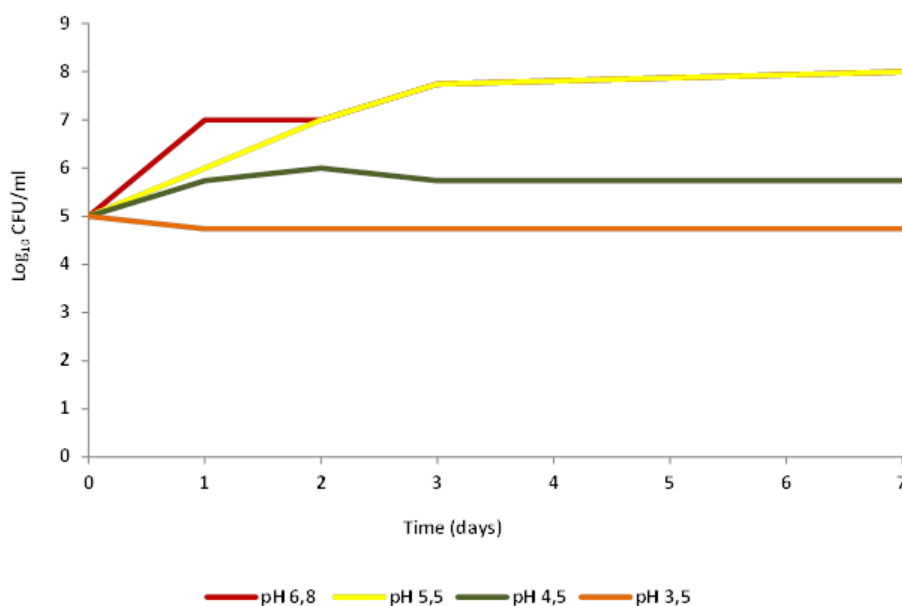


Figure 4.9. Effect of pH on survival of *M. tuberculosis* H37Rv. Cells, at a concentration of 10^5 , were incubated in MB7H9, without Tween 80, at pHs from 6.8 to 3.5 during 7 days. CFU was determined with BACTEC MGIT 960 as described in Materials and Methods section.

However, the combination of low pH on the phagosomal lumen with other antimicrobial factors such as acid-dependent lysosomal hydrolases may render MTB far more sensitive to the killing by the host cell. Therefore, we measured the activity of cathepsin B, a lysosomal cysteine protease, which enters in the phagosome early in its maturation (Sturgill-Koszycki et al., 1996). Increased activity of cathepsin B was detected in cultures treated with the compounds (Figure 4.10). When compared with the untreated control cultures, cathepsin B activity was induced by 21% by VP, 42% by TZ, 33% by CPZ and FPX, and 32% by HAL, in average regardless the macrophage is infected or not infected. These observations indicate that the inhibitors prompted the acidification of the phagosomes containing MTB and thus enhanced the proteolytic activity of cathepsin B. These results show that phagosome acidification is important for the function of degradative hydrolases as well other phagosomal functions, as previously suggested (Vandal et al., 2008).

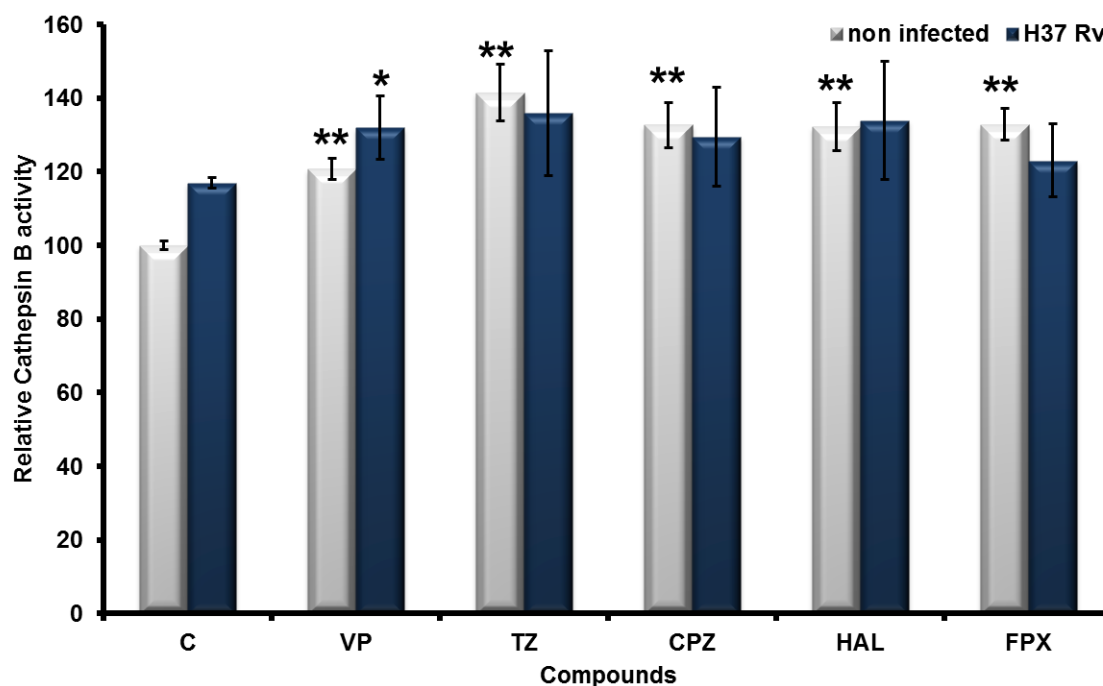


Figure 4.10. Determination of cathepsin B activity.

Human monocyte-derived macrophages were treated with verapamil, thioridazine, chlorpromazine, flupenthixol, and haloperidol. Cathepsin activity was evaluated also with cultures infected with *M. tuberculosis* H37Rv and treated with the compounds. VP was tested at 10 µg/ml; TZ at 2.5 µg/ml; CPZ at 1.25 µg/ml; HAL at 1.25 µg/ml; FPX at 1.25 µg/ml. INH: isoniazid; VP: verapamil; TZ: thioridazine; CPZ: chlorpromazine; HAL: haloperidol; FPX: flupenthixol (* $p < 0.05$; ** $p < 0.01$).

4.4.9. Multidrug resistant *M. tuberculosis* strains demonstrate enhanced fitness.

We observed that the activity of the compounds was influenced by the genotype and fitness of the strains. Accordingly, we characterized the genotype of these strains and evaluated the growth patterns *in vitro* and *ex vivo* as indicators of fitness. Five strains were found to belong to LAM genotype: two belonging to cluster Q1, two from cluster Lisboa3, and one non-clustered strain; one Beijing strain, and one strain with a non-defined genotype (Table 4.1). This strain was further analyzed at SITVITWEB database and the corresponding MIRU12-VNTR pattern was designated as VNTR international type (VIT) number 21. Next, we determined the growth rates for all strains grown in liquid media. First, we examined the time necessary for the strains to adapt to a new environment in liquid culture starting with a well-defined inoculum. In general, except strains

286/09 and 69/11, all grow slightly faster when compared with the control strain, H37Rv (Figure 4.11). The isolates 286/09 and 69/11 grow relatively slower than all others strains. Second, we evaluate the growth rate, *i.e.*, the time necessary to reach 400 GU (the acceleration phase). All strains demonstrated similar growth rates, except strains belonging to cluster Q1 (149/09 and 29/12) and the isoniazid resistant strain 294/09. We observed that strains belonging to cluster Q1 take more time to reach the same growth units than strains from cluster Lisboa3 and Beijing, and less than H37Rv (Figure 4.11). Strain 294/09 showed a dramatically slower replication rate when compared with the other strains with the same mutation. Whilst 294/09 is resistant just to isoniazid, the other strains are multidrug resistant. Collectively, the results show that multidrug resistant strains included in this study have a similar ability to adapt to a new environment but differ in their growth rates *in vitro*.

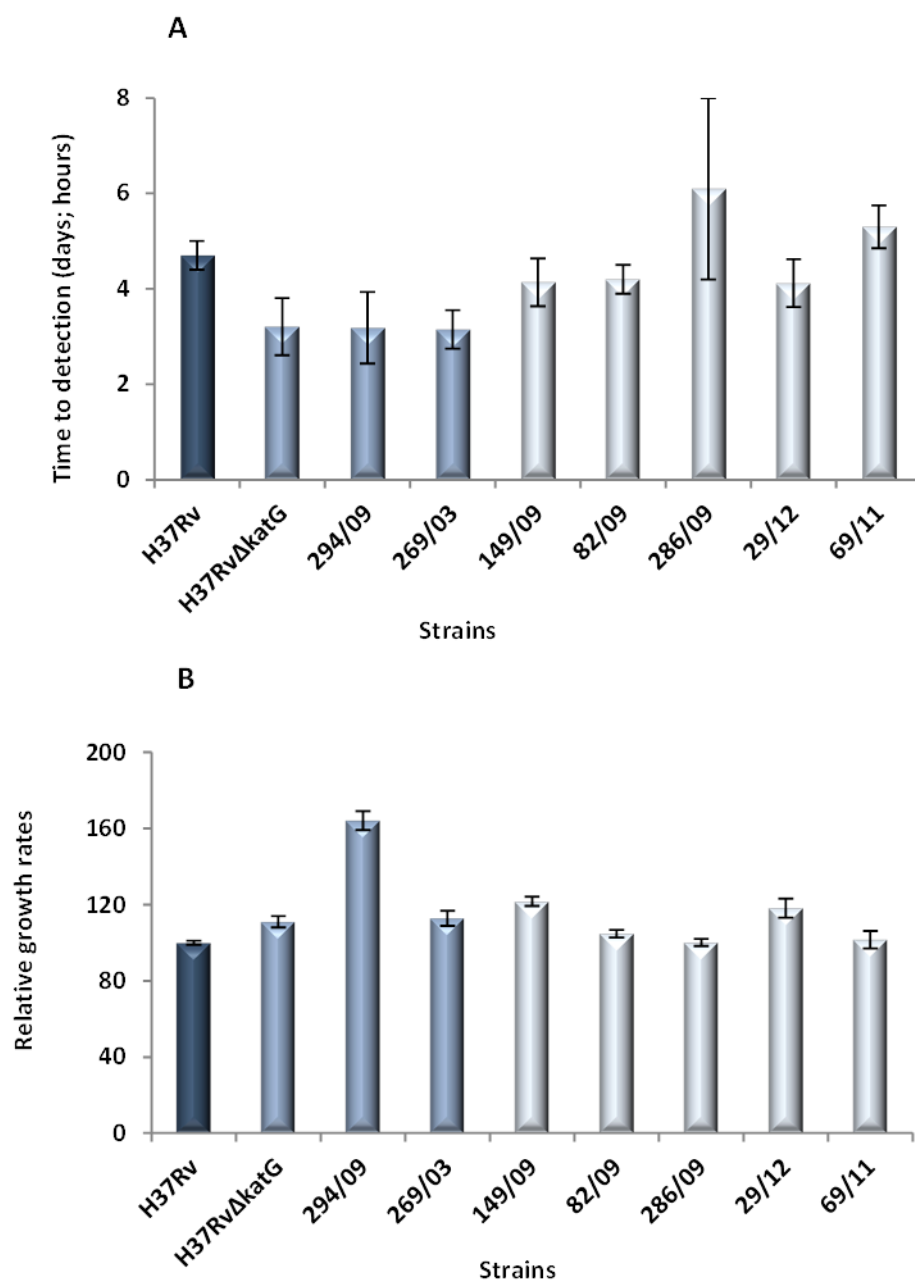


Figure 4.11. *In vitro* determination of the fitness of the *M. tuberculosis* strains. A) Comparison between the capacities of the strains to adapt to a new environment measured as the time to reach the positivity threshold of 75 GU; B) Relative growth rates determined as the ratio between the growth rate of each strain and the growth rate of the control strain. The growth rates which were based on the time to detection between 75 and 400 GU.

Next, we evaluate the ability of some of these strains to grow inside macrophages as a surrogate of intracellular fitness. For that, human monocyte-derived macrophages were infected with multidrug resistant strains belonging to cluster Lisboa3 and Q1, the isoniazid monoresistant strains 294/09, 269/03, and the H37Rv *katG* deletion mutant. As a control, we used the H37Rv reference strain. We found that the MTB multidrug resistant strains have enhanced fitness when compared with susceptible or monoresistant strains. As can be observed in Figure 4.12, both strains grew more quickly in macrophages than all other strains. At the end of five days, the number of viable bacteria was significantly higher than the one reached by the susceptible strain H37Rv. Moreover, H37Rv was able to replicate much faster than the isoniazid monoresistant strains. Regarding H37Rv Δ *katG*, this strain is highly resistant to isoniazid *in vitro* and does not have catalase activity (data not shown). We observed that this strain have reduced ability to survive in human macrophages. However, *katG* deletion mutants can cause disease in humans. One possible explanation is that the bacteria can acquire compensatory mutations to resist to oxidative burst *in vivo* (Sherman et al., 1996). We also observed a reduction of the fitness of the strain with mutation *katG*315. Strain 269/03 retains catalase activity and demonstrates a physiological cost inside macrophages. *katG*315 is the most common isoniazid resistance-conferring mutation found in clinical settings. Although, it is assumed that strains with *katG*315 can maintain *katG* activity and virulence in mice without significant loss of fitness (Pym et al., 2002b), others show that the MTB strains with *katG*315 mutation are less fit on murine macrophages (Rey-Jurado et al., 2011). It has been shown that the genetic background of a strain influences the fitness of drug-resistant MTB (Gagneux et al., 2006). Altogether, it thus seems that intracellular fitness of the MTB strains varies according to their resistant pattern and the mutations associated and the multidrug resistant strains studied showed an increased replication rate inside macrophages indicating a higher fitness for strains with this genotype. Furthermore, overexpression of efflux pumps was more evident on multidrug resistant strains than in isoniazid monoresistant strains and the compounds shown to be more effective on the multidrug resistant strains. From these results we establish association between drug resistance, the genetic background and overexpression of efflux pumps.

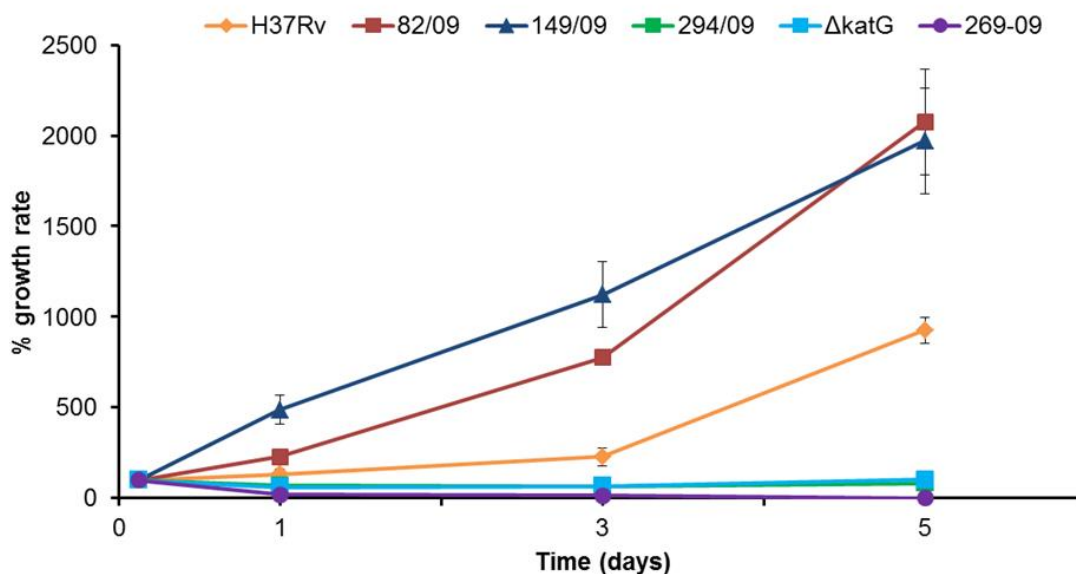


Figure 4.12. Growth rate of *M. tuberculosis* strains in human monocyte-derived macrophages during a 5 days period. CFU were determined after 3h and 1, 3 and 5 days post infection.

4.4.10. Ion channel blockers increase the viability of MTB-infected human macrophages

In order to evaluate the cytotoxicity of the inhibitors in human macrophages we measured their metabolic activity by quantifying the reduction of resazurin to resorufin through fluorescence emission. Our results show that the inhibitors possess different cytotoxic profiles (Figure 4.13A), measured after 3 days of treatment, with CPZ and FPX being the most toxic by showing a reduction of more than 50% of the viability with 5 $\mu\text{g/ml}$ treatment, and VP the less toxic displaying no significant toxicity in concentrations as high as 10 $\mu\text{g/ml}$. Overall the concentrations used for the intracellular killing of mycobacteria and for promoting acidification of intracellular vesicles resulted in no decrease in macrophage viability. In fact, during the infection with H37Rv, the inhibitors actually improved macrophage viability (Figure 4.13B) by almost approximately 20%. From these results we conclude that small concentrations of the inhibitors not only enhance mycobacterial killing and promote vesicle acidification and proteolysis, they also improve the viability of MTB-infected macrophages.

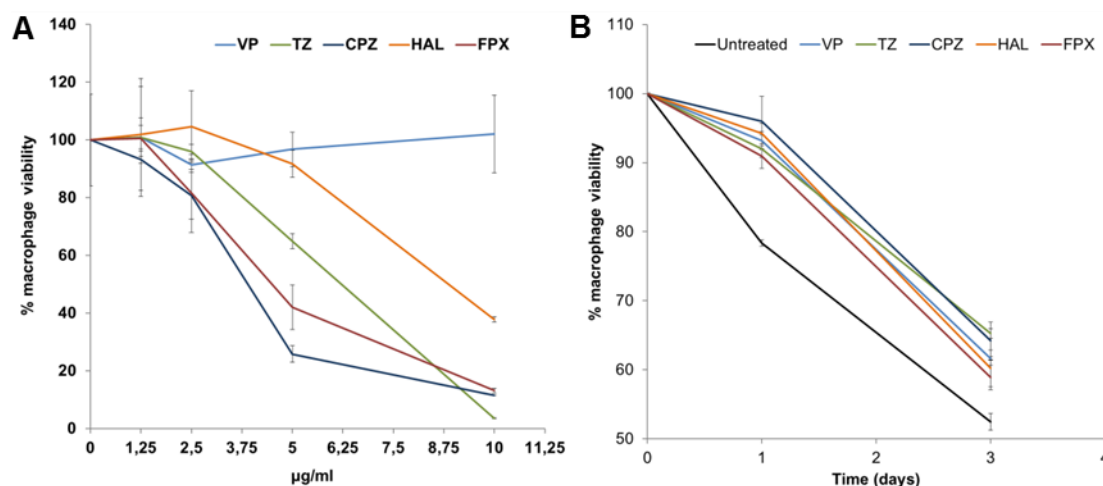


Figure 4.13. Effect of the ion channel blockers on macrophage viability. Macrophage viability was measured by fluorescence quantification of resazurin reduction in uninfected macrophages (A) after 3 days of treatment with several concentrations of the inhibitors or in H37Rv infected macrophages (B) treated with VP at 10 µg/ml; TZ, 2.5 µg/ml; CPZ, 1.25 µg/ml; HAL, 1.25 µg/ml; FPX, 1.25 µg/ml. VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; HAL, haloperidol; FPX, flupenthixol. Values are depicted relative to uninfected untreated control.

4.5. Discussion

The global emergence of multi- and extensively drug resistant tuberculosis has hampered the perspectives of tuberculosis control and elimination. Multidrug-resistant tuberculosis requires a prolonged treatment with second line drugs that are less effective and more toxic. In addition, for extensively drug resistant tuberculosis the chances to achieve treatment success are further reduced. The therapeutic options for these patients can be limited to second-line oral bacteriostatic agents and to drugs with questionable reliability and reproducibility known as WHO Group V (WHO, 2008). Therefore, there is an urgent need for the identification of compounds with different mechanisms of action that can be used in combination with the existing anti-tuberculosis drugs in order to shorten the treatment. Some new drugs have been developed in the latest years and they demonstrated potential to achieve this objective (Rivers and Mancera, 2008). However, they are still limited in number emphasizing the need for new approaches. In this study, we provide evidence that VP, FPX, TZ, CPZ, and HAL act synergistically with the main first line antibiotics to restrict MTB growth either drug susceptible or resistant strains. However, they were not able to restore full drug susceptibility. Nevertheless, they substantially assist in

the reduction of the resistance level to the antibiotics and, by this manner, potentiate their activity. This evidence was confirmed by time-kill studies through which we were able to demonstrate that these compounds are capable to prevent the emergence of resistance and preserve the activity of the co-administered antibiotics. Besides, the low mutation frequencies determined for each ion channel blocker tested (10^{-10} - 10^{-11}) indicate that they are less subject to acquire spontaneous mutations for resistance. Assuming that the MTB burden in the lungs is around 10^8 - 10^9 and the mutations rates for isoniazid and rifampicin are approximately 10^{-8} and 10^{-10} (David, 1970), respectively, the ion channel blockers when combined with anti-tuberculosis drugs currently in use, will reduce the probability of development of spontaneous mutations during tuberculosis drug therapy.

At this moment we do not know the exact mechanism or the assembly of mechanisms by which these inhibitors act as antimicrobial agents against MTB. We observed that all compounds lead to a significant decrease in the intracellular mycobacterial load as result of phagosome acidification inducement and enhanced activation of lysosomal hydrolases. Our previous research has lead us to propose that the enhancement of the killing activity of macrophages by these type of compounds was dependent upon the availability of potassium and calcium ions (Amaral et al., 2007; Martins et al., 2008; Viveiros et al., 2012). The inhibition of the transport of calcium and potassium on the phagosome promote the accumulation of these ions, by diffusion from the cytoplasm into the phagosome. The role of calcium in phagocytosis is controversial, yet in the specific case of phagosome maturation, there are considerable evidences showing the need of cytosolic calcium increase for this process to occur (Nunes and Demarex, 2010). In fact, several studies have shown that in MTB-infected macrophages this rise in calcium levels fails to occurs, correlating with the arrest of phagosome-lysosome fusion (Malik et al., 2000, 2001, 2003; Thompson et al., 2005). Moreover, phagosomal acidification due to increased V-ATPase expression was recently proposed to be negatively controlled by the Abelson (Abl) tyrosine kinase (Bruns et al., 2012). Tyrosine kinase expression is dependent on calcium influx, partially through L-type channels (Raymond and Millhorn, 1997), thus the impact of calcium on tyrosine

inhibition can be associated with a decrease in the intracellular ATP availability and the increase of the expression of V-ATPase. This increase of phagosome acidification and calcium concentration in the cytosol would altogether result in phagosome maturation and enhanced bacterial killing (Bruns et al., 2012; Napier et al., 2011).

Our results *in vitro* show that the ion-channel blockers are good anti-mycobacterial agents, especially when used in combination with anti-tuberculosis drugs, indicating that they interfere directly with the bacteria. In eukaryotic cells it is known that these drugs act by blocking receptors for neurotransmitters such as dopamine and their neuroleptic activity is attributed to their role on the mitochondrial electron transport chain; HAL and CPZ inhibit the mitochondrial NADH: ubiquinone oxidoreductase (complex I) (Balijepalli et al., 1999). In MTB it is believed that phenothiazines also inhibit one of the key enzymes of the mycobacterial respiratory chain, the type II NADH: menaquinone oxidoreductase (NDH-2) (Warman et al., 2013; Weinstein et al., 2005), also called as alternative NADH dehydrogenase (Kerscher, 2000). MTB possesses two NADH dehydrogenases: the NDH-1, encoded by the *nuo* operon, which is non-essential in MTB (Rao et al., 2008) and, the non-proton-translocating NDH-2, encoded by the *ndh* gene, which is essential for MTB survival (Miesel et al., 1998). NDH-1 is an equivalent of mitochondrial complex I, while NDH-2 is a single subunit enzyme with NADH/quinol oxidoreductase activity (Yano et al., 2006). In MTB, energy generation is mainly performed by NDH-2 (Rao et al., 2008) whereas NDH-1 is mostly implicated on anti-apoptotic activity of host cells and, not on its original function of generation of energy (Velmurugan et al., 2007). Rao et al. (2008) established a link between NDH-2 and the proton motive force (PMF) through the demonstration that the inhibition of NDH-2 by TZ dissipates the membrane potential. Since TZ and CPZ act as partial uncouplers of oxidative phosphorylation in mitochondria (Modica-Napolitano et al., 2003) and besides their inhibitory effect on NDH-2 they also act as uncouplers of oxidative phosphorylation in *Staphylococcus aureus* (Schurig-Briccio et al., 2014), it is expected that they indirectly promote the collapse of PMF. Uncouplers of oxidative phosphorylation are able to transport protons across biological membranes (Gage and Neidhardt, 1993). By

transporting protons down the electrochemical potential gradient, these agents collapse PMF. The collapse of PMF has detrimental effects on the bacterial cell such as increased production of reactive oxygen species (ROS), inhibition of ATP synthesis (without affecting the respiratory chain and ATP synthase), and disruption of calcium homeostasis.

All the compounds tested were able to promote intracellular accumulation of ethidium bromide, a broad substrate for PMF dependent efflux pumps, in both drug susceptible and resistant strains. Hence, we can conclude that ethidium bromide accumulation promoted by the ion channel blockers is a result of efflux pump inhibition caused by dissipation of PMF. This was previously shown in our studies with susceptible and antibiotic-adapted MTB strains (Machado et al., 2012) and in this study it is now demonstrated, for the first time, on MTB multidrug resistant clinical strains. Moreover, we showed that several efflux pumps are induced upon exposure to isoniazid and rifampin in these multi- and extensively drug resistant clinical strains. We have detected a general and marked expression of almost all efflux genes tested, which is consistent with a stress response to a hostile environment. Afterwards, we sought to see if some of these pumps are also expressed within the macrophage. We found that some of the efflux genes were expressed inside macrophage upon drug exposure mimicking the inducible adaptative response seen *in vitro*. Using the zebrafish larval model, Adams et al. (2011) demonstrated that drug-tolerant bacteria originate in macrophages dependent on the inducible overexpression and activity of bacterial efflux pumps. In their model a bacterial subpopulation tolerant to drugs arise after few days post-infection, in response to host defenses, by the inducement of bacterial efflux pumps, that rendered the bacteria tolerant to several anti-tuberculosis drugs (Adams et al., 2011, 2014), further strengthening the importance of these findings to the design of rational strategies to prevent the emergence of drug resistance in MTB. Furthermore, during infection, pathogens like MTB have developed strategies to neutralize NADPH oxidase function and inhibit phagosomal generation of ROS. Velmurugan et al. (Velmurugan et al., 2007) identified *nuoG* gene of MTB, which encodes a subunit of the NADH dehydrogenase type I, as an anti-apoptotic gene in MTB. Therefore, a complete functional NDH-1 complex

mediates the anti-apoptotic properties of the bacteria (Velmurugan et al., 2007). We hypothesize that the disruption the membrane potential triggered by the compounds, will lead to the impairment of the respiratory chain, promoting apoptosis during infection. It is not clear whether the inhibition promoted by the compounds is due to their interaction with specific target or is just a consequence of the dissipation of membrane potential. We have tried to obtain mutants for these compounds in an attempt to identify their target. So far, we were unable to isolate such mutants. The reason for this can be related with the reduced mutation rates for these compounds and also with essentiality of their putative targets as it is the case of *ndh* and the phenothiazines. Further analyses are under way to explore this subject.

These compounds have been used for years as antipsychotics and anti-hypertensives, are affordable and, can be taken as oral formulation. Several other drugs whose mechanism of action is known to affect mycobacterial metabolic energy and respiration are now in the pipeline. These include bedaquiline, an ATP synthase inhibitor (de Jonge et al., 2007b; Koul et al., 2007), and PA-824 and delamanid, which targets cytochrome oxidase (Manjunatha et al., 2009). although HAL, TZ and CPZ are widely used for the treatment of psychosis, these compounds have significant toxic effects on mitochondrial bioenergetics function, which may result in serious tardive dyskinesia and Parkinson-like disease (Balijepalli et al., 1999; Modica-Napolitano et al., 2003) and cardiotoxicity (Glassman and Bigger, 2001). As such, caution should be taken when design new compounds that target the respiratory chain, since that due to the impairment of the oxidative phosphorylation, mitochondria became unable to meet the energetic needs of cell. On the other hand, VP is shown to be extremely well tolerated with few side-effects documented (Singh et al., 1978). The significant differences observed in the effective concentrations of the ion-channel blockers required to inhibit MTB *in vitro* versus that needed to produce similar effects on macrophages indicates an interaction that involves the biochemical mechanism underlying the inhibitory effect of the drugs within the bacteria, the ability of the macrophages to concentrate the compounds (Ordway et al., 2003) so they can reach therapeutic concentrations, and the consequences of the stimulation of

macrophage bactericidal mechanisms, which altogether result in the elimination of the bacteria. The activity of VP, TZ, CPZ, FPX, and HAL against drug resistant MTB at concentrations usually reached in human serum when they are employed clinically was demonstrated. The results presented in this study enable us to propose the following mechanism of action for these compounds: a) in the bacteria: after entering the cell the compounds will generate a cascade of events which starts with the inhibition of the respiratory chain complexes, though we cannot say at the present moment if the respiratory chain is a direct target. The inhibition of the respiratory chain will lead to i) dissipation of membrane potential; ii) reduction of ATP levels; iii) ROS generation; iv) increase in intracellular calcium levels; and v) cell death; b) on the host cell, the treatment with the inhibitors will result in phagosome acidification that will synergize with several components of the host immune response, such as lysosomal hydrolases and reactive oxygen and nitrogen species leading to bacterial growth restriction. Furthermore the blocking on ion channels will stabilize the MTB infected cells (Mishra et al., 2010) consequently leading to lower necrotic cell death of infected macrophages and lower escape of intracellular bacilli from the hydrolytic activity of the phagolysosome.

Ongoing work aims to test these hypotheses and determine the exact mechanism by which these compounds can eliminate MTB. Furthermore, it will be interesting to evaluate the impact of the genotype of the strains studied on their capacity to trigger the mechanisms described above. MTB strains of the Latino-American-Mediterranean (LAM) genotype, specifically the Lisboa3 and Q1 clusters, is the main genotype circulating in Lisbon (Perdigão et al., 2008). Our study provides evidence that strains from both clusters interact differently with the host, when compared with the other strains. Strains from Lisboa3 and Q1 clusters demonstrated enhanced fitness inside macrophages. Additionally, the experimental procedures used in this study can be applied to characterize and evaluate the bactericidal activity and efficacy of any combination of compounds and antibiotics. In conclusion, our study should provide valuable information for the improvement of known drugs and for the design and development of new ones. Such increased knowledge will allow for better

epidemiological predictions and assist in the development of new therapeutic strategies to fight drug resistant tuberculosis.

4.6. Acknowledgments

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**Chapter 5: The outer membrane pore
protein CpnT of *Mycobacterium
tuberculosis* mediates susceptibility to nitric
oxide and drugs**

Olga Danilchanka, **David Pires**, Elsa Anes, and Michael Niederweis

In this chapter, I contributed in the design and execution of all the macrophage/bacteria interaction experiments as well as to the analysis and discussion of the results and the writing of the manuscript.

The outer membrane pore protein CpnT of *Mycobacterium tuberculosis* mediates susceptibility to nitric oxide and drugs

Olga Danilchanka, David Pires, Elsa Anes, and Michael Niederweis

5.1. Abstract

The cell envelope of *Mycobacterium tuberculosis* contains an unusual outer membrane of low permeability protecting the bacteria from toxic molecules. Yet, most proteins that functionalize this membrane are unknown. Recently, we identified CpnT as an outer membrane channel protein of *M. tuberculosis* that is required for efficient nutrient uptake and for survival in macrophages. In this study, we show that the loss of CpnT conferred high level resistance of *M. bovis* BCG to multiple drugs and nitric oxide. Thus, CpnT is not only utilized for nutrient uptake, but also constitutes the first outer membrane susceptibility factor in slow-growing mycobacteria. This dual function of CpnT is reflected in macrophage infection experiments: While loss of CpnT was detrimental for *M. bovis* BCG in human macrophages that enable bacterial replication, presumably due to inadequate nutrient uptake, it conferred a survival advantage in mouse macrophages that mount a strong bactericidal response. Expression of the pro-inflammatory genes IL-1 β and iNOS in mouse macrophages was lower providing an additional mechanism for the increased survival of the *cpnT* mutant. Expression of *mspA* did not rescue the growth defect of the *cpnT* mutant even in growth-permissive human macrophages although full complementation of all *in vitro* phenotypes was observed. This indicated that the CpnT channel mediates sufficient nutrient influx, but is less accessible to toxic host immune factors than the MspA channel. Taken together, our results provide evidence that the CpnT channel constitutes an outer membrane gateway controlling the influx of nutrients and toxic molecules into *M. tuberculosis*.

5.2. Introduction

Almost 10 million new cases of tuberculosis (TB) are reported each year (WHO, 2013a). Although current TB chemotherapy cures up to 95% of patients within six months (Koul et al., 2011), concerns about a global TB epidemic are growing due to an increasing prevalence of multiple drug-resistant (MDR) and extreme drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (Udwadia, 2012). Thus, development of new drugs that are active against drug-resistant *M. tuberculosis* strains and that can shorten duration of TB chemotherapy is highly desirable. However, the low permeability of *M. tuberculosis* towards the vast majority of small molecules, and the poorly understood transport processes across the mycobacterial cell envelope (Niederweis, 2008a), render most *in vitro* screening approaches ineffective and hamper the discovery of new TB drugs (Koul et al., 2011). Despite numerous genomic, proteomic and metabolomic studies of *M. tuberculosis* physiological processes such as transport across the mycobacterial cell envelope are poorly understood (Niederweis, 2008a). Although mycobacteria are classified as Gram-positive bacteria, their cell envelope consists of a complex macromolecule in which an outer membrane of low permeability is covalently linked to the peptidoglycan-arabinogalactan copolymer (Hoffmann et al., 2008; Niederweis et al., 2010). Outer membrane integrity is required for protection of *M. tuberculosis* from toxic compounds (Brennan and Nikaido, 1995), and is essential for virulence and pathogenicity of *M. tuberculosis* (Barry, 2001). Additionally, nutrient molecules must be able to permeate the outer membrane to sustain *M. tuberculosis* viability and replication. Uptake of small, hydrophilic solutes across the outer membrane of Gram-negative bacteria is mediated by water-filled protein channels called porins (Zeth and Thein, 2010). Influx of solutes through pore proteins is driven by a concentration gradient and primarily depends on the size, charge, hydrophobicity, and shape of the solute (Nikaido and Rosenberg, 1983). Many drugs and toxic solutes exploit the porin channels to enter bacterial cells (Pagès et al., 2008). It has been shown that channel-forming proteins exist in slow-growing mycobacteria such as *M. tuberculosis*, but their low channel activity prevented their identification (Kartmann et al., 1999; Lichtinger et al., 1999).

MspA is the best characterized outer membrane channel protein of mycobacteria. However it is present only in fast-growing species such as *M. smegmatis* (Niederweis, 2008b). MspA is, to date, the only mycobacterial protein whose β -barrel structure is consistent with that of an integral outer membrane protein (Faller et al., 2004), and is widely used as an outer membrane marker protein in mycobacteria (Bansal-Mutalik and Nikaido, 2011; Feltcher et al., 2013; Rezwan et al., 2007). MspA is the major porin of *M. smegmatis* (Stahl et al., 2001), and mediates diffusion of small, hydrophilic solutes across the outer membrane both in *M. smegmatis* (Stephan et al., 2005) and upon heterologous overexpression in *M. tuberculosis* (Mailaender et al., 2004).

Recently, we identified CpnT as an outer membrane protein in *M. tuberculosis* (Danilchanka et al., 2014). CpnT consists of two domains: The oligomeric N-terminal domain forms an outer membrane channel and is required for efficient glycerol uptake. The toxic C-terminal domain is cleaved, secreted and induces necrosis of eukaryotic cells by a currently unknown mechanism. Thus, CpnT promotes replication of *M. tuberculosis* in macrophages probably by increasing nutrient uptake, and escape from the macrophage by inducing a necrosis-like cell death through the secreted toxin (Danilchanka et al., 2014). The domain organization of CpnT and its function in secreting its own C-terminal domain resembles autotransporters in Gram-negative bacteria (Leyton et al., 2012). It was proposed that the N-terminal domain remains in the outer membrane as an open channel after cleavage and secretion of the C-terminal toxin. Such a functional role of the β -domain of autotransporters in nutrient uptake has not been observed in Gram-negative bacteria, most likely because their outer membranes contain up to 1,000-fold more porins as compared to *M. tuberculosis* (Mailaender et al., 2004).

While the function of the CpnT pore domain in glycerol uptake has been established (Danilchanka et al., 2014), the involvement of this protein in other transport processes across the outer membrane of *M. tuberculosis* has not been examined yet. In this study, we showed that the CpnT channel is exploited by a multitude of chemically very different antibiotics and TB drugs, and also by toxic immune host factors such as NO to enter the mycobacterial cell. Thus,

CpnT constitutes the first outer membrane susceptibility factor of *M. bovis* BCG and *M. tuberculosis*. While CpnT is required for replication of both mycobacteria in permissive macrophages, the presence of CpnT is detrimental for *M. bovis* BCG in macrophages with a strong bactericidal response. This implies that *cpnT* expression and the channel activity must be well coordinated to fine-tune the outer membrane permeability to enable sufficient nutrient influx and to limit exposure to toxic compounds.

5.3. Materials and Methods

5.3.1. Chemicals and Enzymes

Hygromycin B was purchased from Calbiochem. All other chemicals were purchased from Merck or Sigma at the highest purity available.

5.3.2. Bacterial strains, media and growth conditions

M. bovis BCG strains (Strain Institute Pasteur) were grown in Middlebrook 7H9 medium (Difco) supplemented with 0.2% glycerol, 0.02% Tyloxapol and 10% OADC (Remel) or on Middlebrook 7H10 plates supplemented with 0.5% glycerol and 10% OADC (Remel). Antibiotics were used when required at the following concentrations: hygromycin (50 µg/ml), kanamycin (30 µg/ml). *M. bovis* BCG strains and plasmids used in this work are described in (Danilchanka et al., 2014). Briefly, ML383 is a wild-type derivative of *M. bovis* BCG with L5 integrated empty vector pML2008 containing hygromycin resistance marker, ML1012 is a transposon mutant of *M. bovis* BCG disrupted with insertion in *cpnT*, ML386 – ML1012 derivative containing pML2008, ML387 – ML1012 derivative complemented with *mshA*, ML388 – ML1012 derivative complemented with *cpnT*, ML805 – ML1012 derivative complemented with *cpnT*_{NTD}.

5.3.3. Antibiotic susceptibility measurements

The minimal inhibitory concentration (MIC) was determined using a microplate Alamar Blue assay (Franzblau et al., 1998) with modifications (Danilchanka et al., 2008a, 2008b). Final drug concentrations were: for isoniazid and ethambutol, 0.03125-1 µg/ml; for streptomycin and rifampicin, 0.04 - 1.28 µg/ml; for levofloxacin, ofloxacin and clarithromycin, 0.0625-2 µg/ml; for p-aminosalicylic acid and moxifloxacin, 0.25-8 µg/ml; for ciprofloxacin and norfloxacin, 0.5-16 µg/ml; for chloramphenicol and cycloserine, 1 -32 µg/ml; for vancomycin, 2.5 - 80 µg/ml; for erythromycin A and tetracycline, 8 - 256 µg/ml; for ampicillin, 30 -960 µg/ml. The Alamar Blue assays were performed twice in triplicate. The nitrate reductase assay was performed to determine MICs of *M. tuberculosis* as previously described (Mojib et al., 2010).

5.3.4. Susceptibility to nitric oxide

M. bovis BCG strains were grown in 7H9/OADC/0.01% Tyloxapol to an OD₆₀₀ of 2. Cultures were pelleted by centrifugation, washed and re-suspended in 1/10 of original volume. A 0.1-ml aliquots of suspensions were added to 0.9 ml of fresh media containing 0 mM, 5 mM, 25 mM and 100 mM sodium nitroferricyanide (III) dehydrate (SNP, Sigma). Each suspension was incubated at 37°C (200 rpm) for 0, 1, 3 or 7 days. Serial dilutions of the cultures were plated on 7H10/OADC/Hyg plates and incubated at 37 °C for 3 weeks, after which colony forming unites (CFU) were counted. The data was recalculated as the percent survival ([number of CFU exposed at day X/number of CFU unexposed at day X] x 100 %). Each strain was tested in triplicate; assay was repeated three independent times.

5.3.5. Macrophage infection experiments.

The human acute monocytotic leukemia cell line THP-1 (ATCC TIB202) and the mouse macrophage cell line J774A.1 (ATTC TIB-67) were maintained as described previously (Jordao et al., 2008). Differentiation of THP-1 monocytes into macrophages was induced overnight with 50 nM phorbol 12-myristate 13-

acetate (PMA). THP-1 cells were infected at a multiplicity of infection (MOI) of 5. In each experiment, three hours after infection, cells were washed three times with PBS to remove non-internalized bacteria. At different time points after infection (3 h, 1, 3, 5 and 7 days) cells were washed with PBS and lysed with 1% Igepal (Sigma) solution in water. Serial dilutions were done in water and plated on Middlebrook 7H10/OADC plates. Colony forming units (CFU) were counted after two weeks of incubation at 37 °C. When required, macrophages were treated with murine IFN- γ (100 IU) overnight before infection.

5.3.6. Statistical analysis

Data are presented as mean \pm standard deviation; *p* values were calculated using the Student's *t* test and a *P* value of <0.05 was considered to be significant.

5.4. Results

5.4.1. Absence of CpnT confers high level drug resistance to *M. bovis* BCG.

The permeability barrier of the outer membrane is a major drug resistance determinant in slow-growing mycobacteria (Danilchanka et al., 2008b). Since lack of porins in Gram-negative bacteria and *M. smegmatis* confers low level resistance to some small, hydrophilic drugs, in particular β -lactam antibiotics (Danilchanka et al., 2008a; Mojib et al., 2010; Nikaido, 2003; Stephan et al., 2004), we hypothesized that channel proteins might be important for uptake of drugs through otherwise impermeable outer membrane of *M. tuberculosis*. Thus far, such proteins are unknown. However, the porin MspA of *M. smegmatis* increases drug susceptibility when expressed in slow-growing mycobacteria (Mailaender et al., 2004). As CpnT was shown to be essential for transport of glycerol in slow-growing mycobacteria (Danilchanka et al., 2014) we thought to test its role for drug resistance. To this end, we determined the drug susceptibility of *M. bovis* BCG *cpnT::Tn* using the microplate Alamar Blue assay. Resistance to small, hydrophilic antibiotics (Table 5.1) was drastically increased in the absence of *cpnT* by up to 32-fold. Resistance to the anti-tuberculosis drugs ethambutol, isoniazid and *p*-aminosalicylic acid (PAS) was increased more than 4-fold. Expression of *mshA* or full-length *cpnT* fully restored the susceptibility of the *cpnT::Tn* mutant (Table 5.1) providing evidence that insertion of a pore protein in the outer membrane is sufficient to mediate susceptibility to these antibiotics, presumably by increasing drug uptake. Remarkably, minimal inhibitory concentrations (MICs) to large and hydrophobic antibiotics such as erythromycin and rifampicin were also increased in the *cpnT::Tn* mutant by at least 16-fold (Table 5.1). The lack of CpnT also made *M. bovis* BCG more resistant to large, hydrophilic antibiotics such as streptomycin and to several fluoroquinolones (Table 5.1). Importantly, expression of both *mshA* and *cpnT* restored the susceptibility of the *cpnT::Tn* strain to wt levels. These results indicate that the outer membrane protein CpnT is an essential drug susceptibility factor of *M. bovis* BCG.

Considering the important role of CpnT in drug susceptibility of *M. bovis* BCG we determined MICs of wt *M. tuberculosis*, the unmarked mutant of CpnT and of the mutant complemented with a full-length or an N-terminal pore domain of CpnT (Danilchanka et al., 2014) using a nitrate-reductase assay (Mojib et al., 2010). We did not detect any significant difference in MICs of tested strains to a variety of drugs and antibiotics (Table 5.2). This result indicated that *M. tuberculosis* has additional outer membrane pore proteins that either do not exist, or are not expressed in *M. bovis* BCG.

Table 5.1. Antibiotic resistance of the *cpnT* mutant of *M. bovis* BCG.

	Antibiotic	MIC for <i>M. bovis</i> BCG (µg/ml)				Resistance factor ^a
		wt	<i>cpnT::Tn</i>	<i>cpnT::Tn</i> + <i>mspA</i>	<i>cpnT::Tn</i> + <i>cpnT</i>	
A	Ampicillin	30	960	60	60	32
	Tetracycline	31.25	>250	62.5	62.5	>8
	Chloramphenicol	4	64	8	8	16
	Ethambutol	0.125	>1	0.125	0.125	>8
	Isoniazid	0.25	>1	0.25	nd	>4
	p-Amino-salicylic acid	2	>8	2	2	>4
	cycloserine	4	8	4	4	2
B	Vancomycin	5	20	10	10	4
	Streptomycin	0.16	0.64	0.32	0.32	4
C	Clarithromycin	0.125	>2	0.5	0.25	>16
	Erythromycin A	16	>256	32	16	>16
	Rifampicin	0.008	>0.128	0.032	0.032	16
D	Ciprofloxacin	1	2	2	2	2
	Ofloxacin	0.25	0.5	0.25	0.25	2
	Levofloxacin	0.125	0.5	0.125	0.125	4
	Moxifloxacin	0.5	1	0.5	0.5	2
	Norfloxacin	1	4	2	2	4

TB drugs and antibiotics were grouped into four classes: (A) small, hydrophilic antibiotics (molecular weight (MW): 102 - 444 g/mol), (B) large, hydrophilic (MW: 484 to 1450 g/mol), (C) large, hydrophobic (MW: 612 - 823 g/mol) and, (D) fluoroquinolones (MW: 319 to 402 g/mol). The minimal inhibitory concentrations (MIC) were measured using the microplate Alamar Blue assay as described (Danilchanka et al., 2008b). (a) The resistance factor is defined as the ratio $MIC_{cpnT::Tn}/MIC_{wt}$.

Table 5.2. Antibiotic resistance of the *cpnT* mutant of *M. tuberculosis*.

Antibiotic	MIC for <i>M. tuberculosis</i> (µg/ml)			
	wt	ΔcpnT	ΔcpnT + <i>cpnT</i> _{NTD}	ΔcpnT +cpnT
Ampicillin	125	125	125	125
Tetracycline	4	4	4	4
Chloramphenicol	2	2	2	2
Isoniazid	0.05	0.05	0.05	0.05
Rifampicin	0.64	0.64	0.64	0.64
Moxifloxacin	0.125	0.125	0.125	0.125
Norfloxacin	2	2	2	2

The minimal inhibitory concentrations (MIC) were measured using the nitrate reductase assay as previously described (Mojib et al., 2010).

5.4.2. CpnT is required for susceptibility of *M. bovis* BCG to nitric oxide.

Next, we examined whether the CpnT channel plays a role in susceptibility of *M. bovis* BCG to other, physiologically more relevant toxic compounds. Nitric oxide (NO) plays a crucial role in control of *M. tuberculosis* infections in mice (MacMicking et al., 1997). Furthermore, previously it was shown that porins mediate susceptibility of *M. smegmatis* to NO and that aquaporin-1 is required for NO permeability in eukaryotic cells (Fabrino et al., 2009; Herrera et al., 2006) suggesting that CpnT of *M. bovis* BCG might have a similar function. To this end, wt *M. bovis* BCG, the *cpnT::Tn* mutant, and the mutant complemented with either *cpnT* or *mshA* were exposed to the NO donor sodium nitroferricyanide (III) dehydrate (SNP), and serial dilutions were spotted on Middlebrook 7H10/OADC agar plates (Figure 5.1A). All strains were killed by SNP in a time-dependent manner (Figure 5.1B). A rapid decrease in viability of the wt and both complemented *cpnT* mutants was observed after 24 hours of exposure, with the majority of bacteria being killed after three days. By contrast, the *cpnT::Tn* mutant grew during the first 24 hours, and was approximately 100-fold more resistant to killing by NO at later time points compared to the wt *M. bovis* BCG and the complemented mutants (Figure 5.1). Thus, CpnT might play an essential role in defense of slow-growing mycobacteria against toxic molecules produced by host immune cells. The observation that the susceptibility of the *cpnT::Tn* mutant to NO is restored by expression of *mshA* indicates that the channel function and/or outer membrane integration of CpnT and other pore proteins mediate NO susceptibility by increasing the permeability

of the outer membrane to this molecule. Furthermore, overexpression of *mspA* in the *cpnT::Tn* mutant resulted in almost complete clearance of the strain after 24 hours of treatment ($8.9 \pm 2.5\%$ survival for the *mspA* expressing strain $173 \pm 75\%$ for the *cpnT::Tn* strain), thus suggesting that MspA-mediated uptake of toxic compounds reduced survival of the strain under adverse conditions.

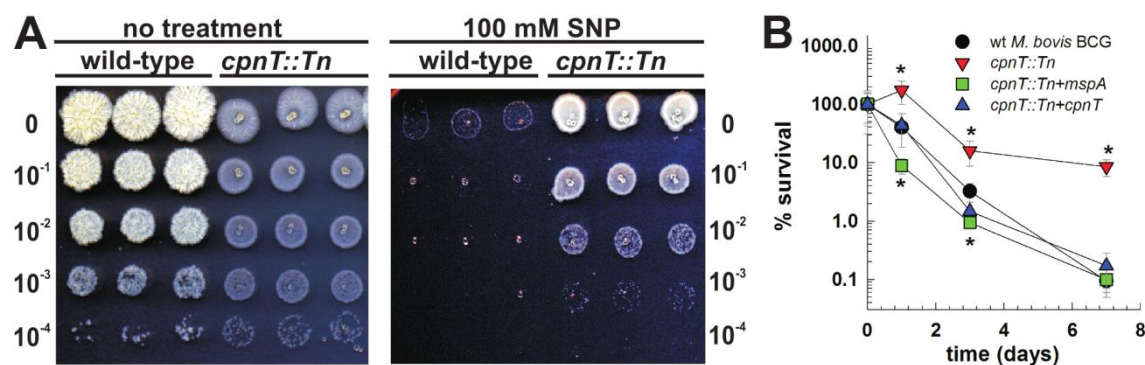


Figure 5.1. Absence of CpnT in *M. bovis* BCG renders high level resistance to nitric oxide.

(A) Survival of wt *M. bovis* BCG and the *cpnT::Tn* mutant after 7 days of exposure to 100 mM SNP. Serial dilutions were spotted on Middlebrook 7H10/OADC agar. (B) Survival of wt *M. bovis* BCG (●), *cpnT::Tn* (▼), *cpnT::Tn* complemented with *mspA* (■), or *cpnT* (▲) after exposure to 100 mM SNP for different times. The colony forming units of the original inoculum of each strain were set as 100% survival. The experiment was done in triplicate and repeated three times. Survival of the wt vs. mutant, or complemented strains was analyzed using Student's t test. * $p < 0.05$ was deemed significant.

5.4.3. The channel-forming N-terminal domain of CpnT is required for growth and survival of *M. bovis* BCG in macrophages.

Previously, we observed that both the channel and the toxin domain of CpnT contribute to the survival of *M. tuberculosis* in macrophages (Danilchanka et al., 2014). This made it difficult to determine the individual roles of these CpnT domains during infection of host cells. *M. bovis* BCG does not permeabilize phagosomes and does not escape from phagosomes, in contrast to *M. tuberculosis* (Manzanillo et al., 2012; Simeone et al., 2012). Thus, the C-terminal toxin domain of CpnT is capable of leaving the phagosome during *M. tuberculosis* infection to induce necrosis in cytosol, while it is likely trapped in phagosomes during *M. bovis* BCG infection where it is incapable to induce

necrosis. This assumption is supported by the virtual absence of cytotoxicity of *M. bovis* BCG or *M. tuberculosis* mutants that lack RD1 region, a primary virulence locus of *M. tuberculosis* that is also missing in *M. bovis* BCG (Hsu et al., 2003; McDonough and Kress, 1995). Hence, we sought to exploit this feature of *M. bovis* BCG to examine the role of the CpnT channel domain in the absence of confounding effects of the toxin domain. Here we show that the absence of *cpnT* drastically reduced intracellular growth of *M. bovis* BCG in the human monocytic cell line THP-1 (Figure 5.1). This is consistent with the results obtained for *M. tuberculosis*-infected THP-1 macrophages (Danilchanka et al., 2014) and underlines the important role of CpnT for survival of slow-growing mycobacteria in macrophages. Growth of *M. bovis* BCG was fully restored by expression of *cpnT*_{NTD} encoding the channel-forming N-terminal domain (NTD) of CpnT. This observation confirmed that the toxin domain is indeed dispensable for normal growth of *M. bovis* BCG in macrophages and that only the pore domain is needed for uptake of solutes and growth. By contrast, CpnT_{NTD} is only capable to restore wt growth of *M. tuberculosis* in THP-1 macrophages during the first 24 hours, while the toxic CpnT_{CTD} is required for normal growth of *M. tuberculosis* at later time points (Danilchanka et al., 2014).

Interestingly, expression of *mspA* did not rescue the growth defect of the *cpnT::Tn* mutant even in the growth-permissive THP-1 cells (Figure 5.2), although full complementation of all phenotypes was observed *in vitro* (Figure 5.1; Table 5.1). These results indicate that the CpnT channel mediates sufficient nutrient influx, but provides a much better protection against toxic host immune factors than the wide open MspA channel (Faller et al., 2004).

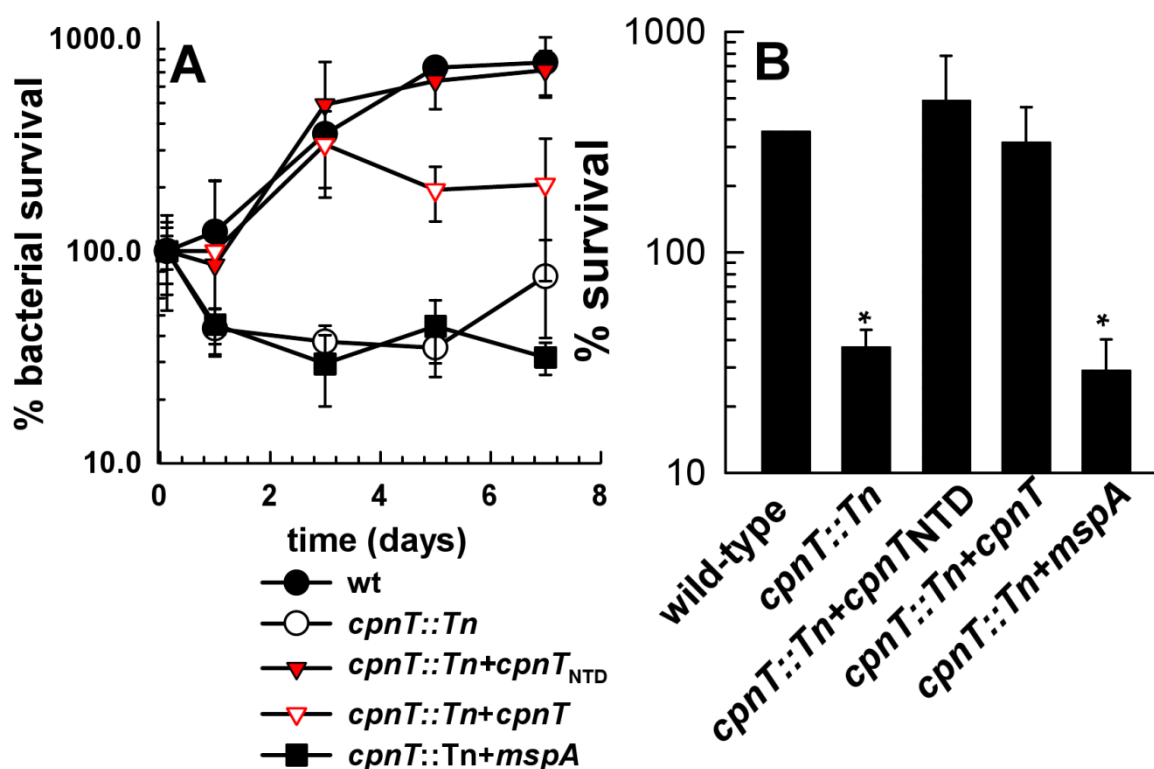


Figure 5.2. Survival of *M. bovis* BCG in THP-1 cells.

A. THP-1 cells were infected with wt *M. bovis* BCG (filled circles), *cpnT::Tn* (open circles), *cpnT::Tn* complemented with *cpnT_{NTD}* (filled triangles), *cpnT* (open triangles) or *mspA* (squares) at an MOI of 5. The colony forming units of the original inoculum of each strain was set as 100% survival. (B) Bar graph representing survival of *M. bovis* BCG in THP-1 cells 3 days post-infection. The colony forming units of the original inoculum of each strain was set as 100% survival. Survival of the wt vs. mutant, or complemented strains was analyzed using Student's t test. * $p < 0.05$ was deemed significant.

5.4.4. The absence of CpnT protects *M. bovis* BCG from killing in macrophages and from the bactericidal response of stimulated macrophages.

We showed that CpnT facilitates uptake of nutrients (Danilchanka et al., 2014), but also enables toxic molecules to enter the bacterial cell (Figure 5.1; Table 5.1). Hence, we wondered which of these properties of CpnT would prevail *in vivo* when the uptake of nutrients for bacterial replication might be less important than protection from toxic compounds, such as NO, which utilize the CpnT channel to enter mycobacterial cells. To this end, we used J774 mouse macrophages in which *M. bovis* BCG does not replicate and is efficiently eliminated (Jordao et al., 2008). We hypothesized that the requirement for

uptake of nutrients through porins under these conditions is minimal, while resistance to toxic compounds produced by macrophages plays a crucial role in clearance of bacteria. As expected, the bacterial burden for all strains decreased during infection of J774 cells (Figure 5.3). However, the *cpnT::Tn* strain was more resistant to killing compared to the wt or complemented strains. Furthermore, stimulation of J774 with interferon- γ , a potent inducer of nitric oxide and other reactive nitrogen intermediates, reduced survival of wt *M. bovis* BCG, but did not affect the *cpnT::Tn* strain (Figure 5.3). These experiments suggest that the inertness of the *cpnT::Tn* mutant to the bactericidal response of macrophages upon interferon- γ stimulation is due to mutant's high level of resistance to NO, a potent effector molecule of mouse macrophages (MacMicking et al., 1997), and possibly other toxic compounds produced by macrophages.

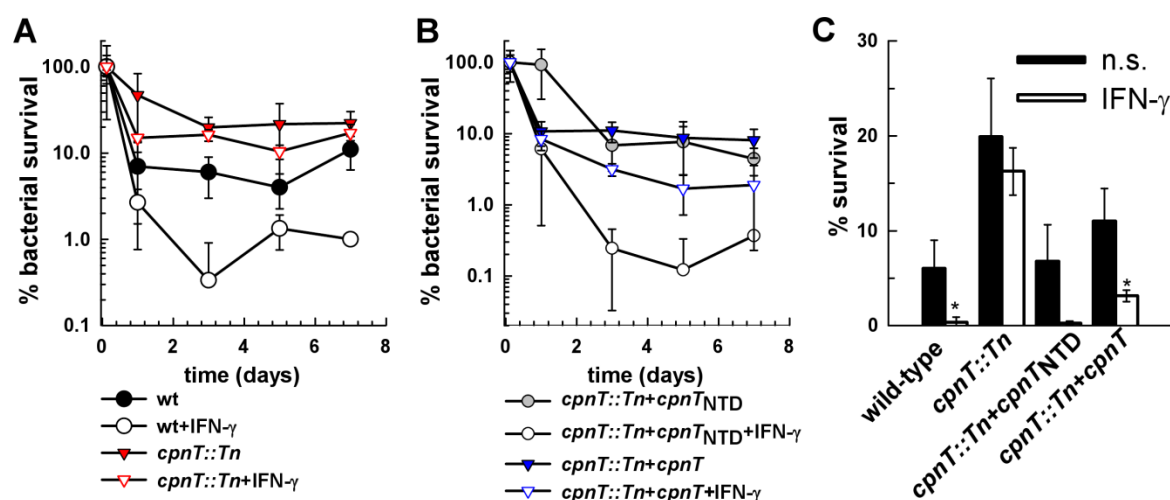


Figure 5.3 Survival of *M. bovis* BCG in macrophages. A, B. J774 cells were infected with wt *M. bovis* BCG (●), *cpnT::Tn* (▼) (B), and the *cpnT::Tn* mutant complemented with *cpnTNTD* (◆), or *cpnT* (▲) (C) at an MOI of 5. When required, J774 cells were treated with murine IFN- γ (100 IU) overnight before infection (open symbols). The colony forming units of the original inoculum of each strain was set as 100% survival. (B) Bar graph representing survival of *M. bovis* BCG in J774 cells 3 days post-infection. When required, J774 cells were treated with murine IFN- γ (100 IU) overnight before infection (open bars). n.s.: non-stimulated J774 cells (filled bars). The colony forming units of the original inoculum of each strain was set as 100% survival. ANOVA one parameter test was performed, with post hoc analysis using Holm-Sidak test. * $p < 0.05$ was deemed significant.

5.4.5. The absence of CpnT reduces the pro-inflammatory response of macrophages infected with *M. bovis* BCG.

In some cases porins are highly antigenic and are recognized by the host immune system (Kuusi et al., 1981; Tabaraie et al., 1994). Consequently, porins can induce inflammation in mice, activation of B cells and release of cytokines (Galdiero et al., 1993; Vordermeier et al., 1987). Thus, an alternative explanation for the increased resistance of the *cpnT* mutant to killing by macrophages might be a lower pro-inflammatory response of macrophages that could result in reduced bacterial killing (Mishra et al., 2010). To test this hypothesis we analyzed the expression of the pro-inflammatory interleukin 1 β (IL-1 β) gene and of the gene encoding the inducible isoform of nitric oxide synthase (iNOS) during infection of J774 mouse macrophages with *M. bovis* BCG. We focused on IL-1 β and iNOS as key markers of *M. tuberculosis* immune responses due to their essential roles for pulmonary infection with this pathogen. Specifically, *M. tuberculosis* infection results in robust initial production of IL-1 in a mouse model of infection, that is followed by the induction of adaptive immune responses including expression of iNOS that produces nitric oxide, a molecule found to be essential not only for antibacterial control, but also for modulation of inflammatory immune responses through alteration of NLRP3 inflammasome (Mishra et al., 2013). We observed two- and five-fold lower iNOS and IL-1 β mRNA levels, respectively, after infection of macrophages with the *cpnT::Tn* mutant compared to the wt strain (Figure 5.4). This difference was independent of the presence of interferon- γ . Expression of IL-1 β and iNOS was partially restored in the *cpnT* mutant complemented by the full-length *cpnT*, and was fully complemented by *cpnT_{NTD}* construct. Thus, lower levels of pro-inflammatory response of macrophages in response to infection with *cpnT::Tn* mutant is likely a contributing factor to its ability to survive better in macrophages with a strong bactericidal response. Taken together, these results suggest that the absence of CpnT confers a survival advantage in the presence of a strong bactericidal response of macrophages to mycobacterial infection, while the nutrient uptake function of CpnT appears to dominate in macrophages under conditions that enable bacterial replication.

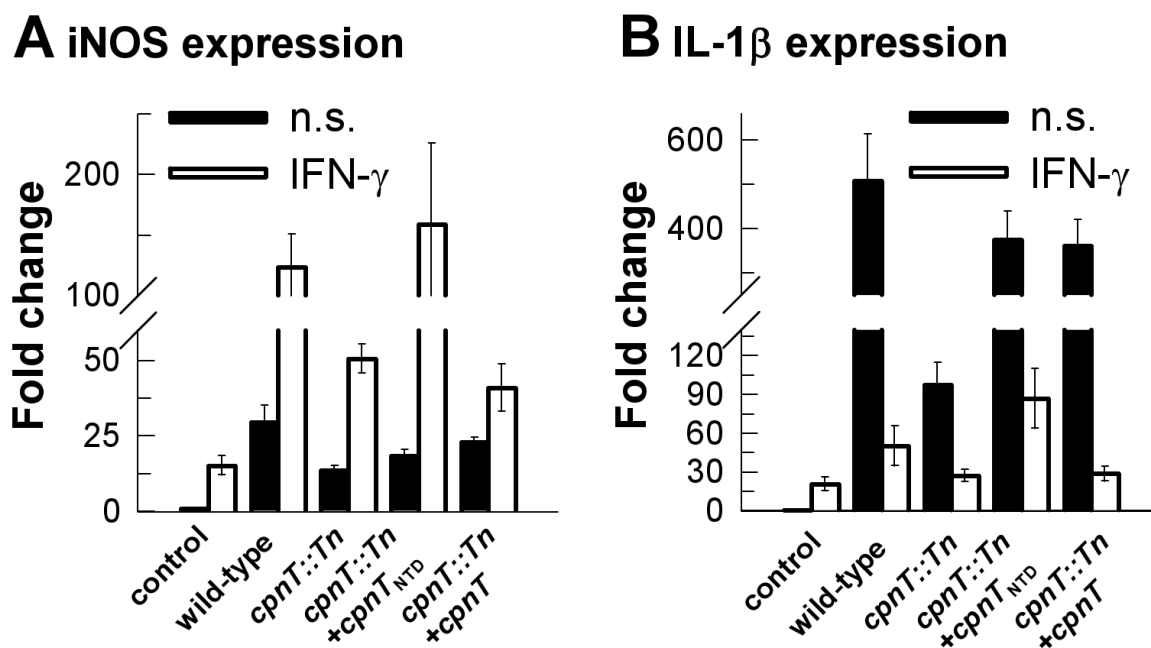


Figure 5.4. Pro-inflammatory gene expression is reduced in the absence of CpnT. qRT-PCR quantification of iNOS (A) and IL- β (B) gene expression in J774 macrophages 24 hours after infection with *M. bovis* BCG strains. Data is represented as the mean \pm SD relative to uninfected cells (control). When required, J774 cells were treated with murine IFN- γ (100 IU) overnight before infection (open bars). N.S.: non-stimulated J774 cells (filled bars).

5.5. Discussion

5.5.1. Role of CpnT in susceptibility of *M. bovis* BCG and *M. tuberculosis* to antibiotics and TB drugs

The outer membrane permeability barrier is of utmost importance for survival of *M. tuberculosis* under harsh conditions *in vivo* (Barry, 2001; Brennan and Nikaido, 1995) and a key component of its intrinsic drug resistance (Jarlier and Nikaido, 1994). In this study we showed that the *cpnT* mutant of *M. bovis* BCG exhibited high level resistance to a wide variety of antibiotics and TB drugs. The resistance factors for most of these compounds were much higher than those previously observed for a single porin mutant of *M. smegmatis* (Stephan et al., 2004), or for Gram-negative bacteria (Nikaido, 2003). In particular, the *cpnT* mutant of *M. bovis* BCG became more resistant to the TB drugs isoniazid and ethambutol, while their MIC's were not affected by the deletion of the main porin gene *mspA* of *M. smegmatis* (Stephan et al., 2004). This difference might be

explained by the larger number of porins in the outer membrane of *M. smegmatis* compared to *M. bovis* BCG. While the number of porins is reduced from 2,400 to 800 by the deletion of *mspA* in *M. smegmatis* (Stephan et al., 2005), no open pores were detected by electron microscopy in *M. bovis* BCG and *M. tuberculosis* (Mailaender et al., 2004). Thus, the loss of CpnT probably has a much larger effect on permeability of *M. bovis* BCG compared with the loss of the porin MspA in *M. smegmatis*. The effect of a single porin deletion in Gram-negative bacteria on drug resistance is even less pronounced as they contain 100-1,000-fold more porins compared to *M. smegmatis* (Niederweis, 2003).

Our results imply that uptake of small, hydrophilic anti-tuberculosis drugs ethambutol, isoniazid, and p-aminosalicylic acid is mediated by pore-forming proteins such as CpnT. Surprisingly, we also found that absence of CpnT in *M. bovis* BCG resulted in high-level resistance to large and hydrophobic drugs such as erythromycin and rifampicin. While we do not assume that these antibiotics diffuse directly through the CpnT pore, deletion of an outer membrane protein may indirectly reduce drug susceptibility. Such a mechanism has been revealed in *M. smegmatis* (Stephan et al., 2004). Deletion of the major porin MspA not only reduced the permeability of *M. smegmatis* for hydrophilic, but also hydrophobic compounds such as chenodeoxycholate and concomitantly increased resistance to large, hydrophobic antibiotics, in contrast to porin mutants of *E. coli* and other Gram-negative bacteria (Delcour, 2009). It has been proposed that integration of proteins interferes with lipid interactions in the outer membrane of mycobacteria and, hence, may increase permeability across the lipid bilayer inversely to its protein content (Stephan et al., 2004). Further experiments utilizing porins with closed channels that can integrate into the outer membrane, but are deficient for solute transport will enable to test this hypothesis.

In contrast to *M. bovis* BCG the lack of CpnT did not increase drug resistance of *M. tuberculosis*. This may indicate that the lack of CpnT is compensated by another pore protein in *M. tuberculosis* that is not present or produced by *M. bovis* BCG. However, to our knowledge, such proteins have not been described yet, but their presence is suggested by previous biochemical characterization of

channel-forming proteins of slow-growing mycobacteria (Kartmann et al., 1999; Lichtinger et al., 1999). Since the genomes of *M. bovis* BCG and *M. tuberculosis* are almost identical except for the so-called regions of difference (RD) that were deleted during attenuation of *M. bovis* BCG (Brosch et al., 2001, 2002), the apparent compensation of the lack of CpnT by *M. tuberculosis* might also result from a different regulation of gene expression in both mycobacterial species (48). The different susceptibility profiles of wt *M. bovis* BCG and *M. tuberculosis* (Table 5.1; Table 5.2) may reflect different pore-mediated outer membrane permeability in both organisms, probably in addition to other factors such as altered gene expression and metabolism (Lofthouse et al., 2013; Pan et al., 2012).

To our knowledge, CpnT has not been previously associated with drug-resistant tuberculosis. However, resistance in approximately 5-30% of clinical isolates (dependent on antibiotic) cannot be explained by mutations in any known genes associated with resistance to a particular drug (Louw et al., 2009) suggesting that other undefined mechanisms are involved. As deletion of *cpnT* in *M. bovis* BCG leads to multidrug-resistant phenotype *in vitro*, we propose that some of *M. tuberculosis* clinical isolates might also have mutations in this gene and other, so far unknown porin genes. Indeed, *cpnT* was recently identified as a gene with a strong, consistent association with drug resistance in a study that looked at 161 *M. tuberculosis* isolates from China with different drug resistance profiles (Zhang et al., 2013a).

5.5.2. Role of CpnT in resistance to nitric oxide and other toxic compounds in macrophages

This study provides evidence that outer membrane proteins of *M. tuberculosis* have a major influence on the resistance to toxic compounds utilized *in vivo* to kill bacteria: The absence of CpnT renders *M. bovis* BCG 100-fold more resistant to nitric oxide compared to the wt strain. This is consistent with previous observations that the MspA porin of *M. smegmatis* mediates susceptibility to nitric oxide in mycobacteria (Fabrino et al., 2009; Purdy et al., 2009), but, to our knowledge, porins of Gram-negative bacteria were never

implicated in this process. This is likely due to the very large number of various channel-forming proteins in the outer membrane of these bacteria (Kessel et al., 1988). Thus, porin mutants in Gram-negative bacteria still have a relatively large number of channels remaining in the outer membrane which often prevents detection of phenotypes in susceptibility and/or transport experiments.

Interestingly, expression of *mspA* did not rescue the growth defect of the *cpnT::Tn* mutant even in growth-permissive THP-1 cells (Figure 5.2), while full complementation of all phenotypes was observed *in vitro* (Table 5.1, Figure 5.1). These results indicate that the CpnT channel mediates sufficient nutrient influx in *M. bovis* BCG, but provides a much better protection against toxic solutes *in vivo* than the wide open and potentially unregulated MspA channel (Faller et al., 2004). Although MspA provides an efficient uptake pathway for nutrients, the apparently unregulated uptake of toxic compounds by MspA leads to reduction of *M. bovis* BCG survival in macrophages. These observations suggest that in order to survive and grow in macrophages a tight balance between nutrient acquisition and minimization of the influx of toxic compounds is essential, and disturbance of this equilibrium in either direction might be detrimental during infection. These results also indicated that increasing the efficiency of channel protein-mediated outer membrane permeability may indeed be an attractive approach for developing new TB drugs with an alternative mechanism of action as proposed earlier (Niederweis et al., 2010).

5.5.3. CpnT constitutes a gateway protein in *M. bovis* BCG and *M. tuberculosis*

The important role of CpnT in susceptibility to drugs and NO implies that it constitutes a gateway protein in the outer membrane of *M. bovis* BCG. This is consistent with the observation that other outer membrane channel proteins control entry of small, hydrophilic solutes including antibiotics into cells of Gram-negative bacteria and mycobacteria (Danilchanka et al., 2008a; Pagès et al., 2008). Not surprisingly, porin mutations often cause drug resistance of clinical isolates of Gram-negative bacterial pathogens. However, it needs to be noted that CpnT does not belong to the group of classical porins as found in Gram-

negative bacteria, whose physiological function is only to mediate influx of small, hydrophilic solutes through their water-filled channels across the outer membrane (Nikaido, 2003). The primary function of CpnT in *M. tuberculosis* appears to be the secretion of a novel necrosis-inducing toxin located on the C-terminal domain of the protein by utilizing its N-terminal domain in an autotransporter-like mechanism (Danilchanka et al., 2014). Although details of this secretion process are unknown, it is clear that the channel-forming activity of the N-terminal domain of CpnT is the sole molecular determinant mediating drug susceptibility and nutrient transport in *M. bovis* BCG. It is interesting that the C-terminal domain does not seem to interfere with the channel activity indicating that perhaps the vast majority of CpnT proteins secrete the toxin and only the N-terminal domain remains in the outer membrane of *M. tuberculosis*. However, since full-length CpnT is also detectable in the outer membrane of *M. tuberculosis*, an alternative explanation for the fact that all resistance phenotypes of the *cpnT* mutant of *M. bovis* BCG are complemented by the CpnT N-terminal domain alone might be that the toxin domain does not interfere with transport through the CpnT channel. In this regard, it would be interesting to determine the ratio of full-length versus cleaved CpnT in *M. bovis* BCG and *M. tuberculosis* under different conditions, in addition to *in vitro* bilayer channel activity studies with a full-length CpnT protein.

5.5.4. The channel activity of CpnT is a double-edged sword for *M. bovis* BCG in macrophages

The macrophage experiments with *M. bovis* BCG revealed dual functions of CpnT. (i) In macrophages that prevent intracellular mycobacterial replication, absence of CpnT confers a survival advantage. The 100-fold increased resistance of the *cpnT* mutant to nitric oxide might play a key role in this regard, although regulatory effects, as evidenced by reduced iNOS and IL-1 β levels, probably contribute to the increased resistance of the *cpnT* mutant to killing in J774 macrophages. (ii) Under *in vivo* conditions that enable bacterial replication, such as in human THP-1 macrophages, the presence of CpnT is beneficial, probably because of increased nutrient uptake. However, the *M.*

tuberculosis cpnT mutant did not exhibit a virulence defect during mouse infection (Danilchanka et al., 2014) in agreement with the classification of *cpnT* as a non-essential gene in transposon site hybridization experiments (Sasseti and Rubin, 2003), and previous observations that glycerol utilization is not required for virulence of *M. tuberculosis* in mice (Pethe et al., 2010). The lack of a virulence defect for the *M. tuberculosis cpnT* mutant could be caused by the presence of an alternative outer membrane pore protein that is expressed in *M. tuberculosis*, but not in *M. bovis* BCG as indicated by the absence of drug resistance phenotypes of the *M. tuberculosis* mutant in this study. Identification of such additional channel proteins will be important to delineate drug and nutrient uptake pathways in the notoriously impermeable cells of *M. tuberculosis*.

5.6. Conclusions

CpnT has multiple functions in nutrient uptake across the outer membrane and susceptibility to toxic solutes due to the activity of its N-terminal domain. The identification of this pore protein represents the first step in understanding outer membrane permeability of *M. tuberculosis*. Knowledge of the structure and the biophysical properties of the CpnT N-terminal channel domain might help to improve drug uptake and, thereby, to overcome the outer membrane permeability barrier, a major hurdle in TB drug development.

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Chapter 6: Conclusions

In this thesis I proposed to address the problem of how *Mycobacterium tuberculosis* manipulates the immune effectors of the endocytic and antigen presentation pathways of macrophages and dendritic cells. These are complex pathways comprehending a vast array of intervening molecules. This thesis only focused on a small, yet significant portion of these pathways, with a particular emphasis on the role of cathepsins and the phagolysosomal environment influencing their activity. Our choice for these molecules was originated from the realization that little attention was given to these enzymes in the context of bacterial infection when compared to cancer and other fields of biomedical research in spite of being implicated on several aspects of innate immunity and initiation of adaptive immunity. The network of processes in which cathepsins are implicated is also vast, being able to directly act on pathogens or indirectly being involved in the processing, maturation and activation of other immune effectors that in their turn will act on the pathogen. For these reasons, this thesis initially focused on cathepsins.

Our first approach was to understand how cathepsins are subject to regulation induced by MTB infection and how does it influence the outcome of infection. Our results have shown that cathepsin regulation is tied to the infecting bacteria and also to the type of cells being infected. Macrophages displayed a complex regulation with different cathepsins being up-regulated or down-regulated in response to infection. Contrariwise, infection of DCs induced the up-regulation of the majority of cathepsins. These differences might reflect the different roles proposed for these cells and contrasting adaptations to those roles by the pathogens. As mentioned in this thesis, while MTB benefits from a reduced initial immune response to increase its burden to the host (depressed local immunity from MTB-infected macrophages) it might also benefit from the translocation of DCs to the lymph nodes and induction of signals resulting in the recruitment of fresh cells to the infection locus. Control of the local immune response is also crucial to avoid inflammatory lesions and the cellular response mediated by macrophages and T cells plays a relevant role in it. We also tested the effects of stimulating macrophages and DCs with IFN- γ or TNF- α , respectively. We did this to simulate the different cellular conditions that MTB might encounter upon infection. Overall, when comparing the phenotypes

induced by the *M. smegmatis* and MTB, our results indicated a common pattern in cathepsin expression. MTB induces the down-regulation of the majority cathepsins or represses their up-regulation. In macrophages, we discovered an endogenous intermediate in this regulatory process for cathepsin S, the micro RNA miR-106b-5p. The up-regulation of this miRNA correlated with the down-regulation of cathepsin S observable 24h upon infection with MTB and we could prove the interaction between these two molecules. The result of cathepsin regulation was the increase of MTB intracellular survival as we showed in our inhibition and silencing experiments. Interestingly the down-regulation of cathepsins B, S and L also lead to an increase of the surface expression of HLA-DR, part of the HLA class II family.

Cathepsins are subject to different forms of regulation and the vacuolar environment where they reside will influence their activity. We proposed that we could influence this environment using pharmacologic molecules already approved for other medical conditions. The common feature found on these molecules was their ability to inhibit ion channel transport activity leading to improved antibiotic treatment when used in combination with first line anti-TB drugs. From these initial evidences we hypothesized that this improved treatment was not only due to the inhibition of drug efflux systems of MTB but also to an increase in macrophage mediated killing mechanisms. Our evidences support the hypothesis that this improvement was due to a general vesicular acidification in host macrophages that not only is known to be a bactericidal response in its own, but as I have reported in this thesis, also induces an increase in cathepsin B activity.

Finally, we focused our attention on the bacteria. In spite of all these mechanism of MTB killing and the improvements we were able to achieve through silencing or chemical inhibition of cathepsins and usage of ion channel blockers to enhance pH-dependent cathepsin activity, the fact is: some of the bacteria are able to survive. We concentrated on the membrane of MTB due to its characteristic and distinguishing features but also because it is the primordial barrier against foreign aggression. Indeed, we saw that restriction of membrane transport caused by the knock-out of the outer-membrane channel CpnT in mycobacteria is a relevant mechanism to enhance their survival when facing the

bactericidal actions of antibiotics or macrophage intracellular release of oxidative species. Furthermore, the lower induction of macrophage pro-inflammatory genes by the CpnT mutant indicated that restriction of outer-membrane transport or restriction of this surface channel complex might also help to avoid triggering innate recognition by the macrophage.

Overall, with this thesis I propose that cathepsins are main effectors and mediators of the innate and adaptive response against MTB and because of their relevance MTB evolved to restrict their activity using the host own mechanisms to increase its intracellular survival. I propose that since the host own mechanisms are being targeted by MTB, we should focus to implement strategies to counter-modulate those mechanisms in favor of the host and combine them with current antibiotic therapy in order to improve the chances of success. I propose two methods for that; (1) the usage of miRNAs or their inhibitors to restore the bactericidal response against MTB; (2) modulation of the phagosome and phago-lysosomal environment using ion channel inhibitors to generate a degradative milieu and an increased proteolytic activity. Lastly, I propose that although membrane transport in MTB is reduced, it still provides a pathway for antibiotics and host bactericidal molecules and by understanding this pathway we may develop drugs that can exploit it to enter the bacilli.